REGULATION OF INTRACELLULAR FREE CALCIUM IN NEURONAL CELLS BY OPIOIDS

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ABSTRACT

Title of Dissertation:	Regulations of Intracellular Free Calcium in Neuronal Cells by Opioids
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The regulation of intracellular free calcium level by opioids was investigated in a sensory neuronal cell line, ND8-47 (neuroblastoma x dorsal root ganglion neuron hybrid cells), and in cultured mouse dorsal root ganglion (DRG) neurons. Receptor binding studies show that ND8-47 cells carry a high density of opioid δ type receptors. In these cells, δ agonists induced a transient increase in intracellular calcium concentration ($[Ca^{2+}]i$) in a concentration-dependent fashion; among them, D-Ser^2-Leu^5-enkephalin-Thr (DSLET) and deltorphin-II were more effective than [D-Pen^{2,5}]enkephalin (DPDPE). This effect was blocked by the non-selective opioid receptor antagonist, naloxone, and the δ-receptor antagonist, naltrindole. The sub-type specific δ-receptor antagonists, 7-benzylidene naltrexone (BNTX; δ_1), and naltriben (NTB; δ_2), were used to characterize further the subtype of δ-receptors that mediated this response. NTB was more potent than BNTX in antagonizing DSLET-induced increase in $[Ca^{2+}]i$. The increase in $[Ca^{2+}]i$ induced by DSLET was blocked by dihydropyridine-sensitive L-type calcium channel blockers, nifedipine (1 μM) or verapamil (1 μM), and was not observed in the absence of external calcium.

The type of G protein mediating this response was analyzed. Pretreatment of ND8-47 cells with pertussis toxin blocked DSLET-induced increase in $[Ca^{2+}]_i$; cholera toxin had no effect. The G protein α subunits, α_{i2}, α_{i3}, α_q and α_s, were detected using Western blots while α_o and α_{i1} were not found in ND8-47 cell membranes. The DSLET-induced increase in $[Ca^{2+}]$i was inhibited by prior treatment of the cells with antisense oligonucleotide directed against G protein α_{i2}-subunit, but not by the complementary sense oligonucleotide, or by antisense oligonucleotides to other G protein α-subunits present in the membranes. It appeared that $G\alpha_{i2}$ was required in the opioid-induced increase in $[Ca^{2+}]$i. The effect of sustained exposure to opioid on receptor binding and function was studied. Opioid-induced increase in $[Ca^{2+}]$i was lost within 1 h of incubation with DSLET (1 μM), but a reduction in receptor number was not observed until after 6 h of DSLET treatment. There appear to be at least two consequences of sustained δ receptor activation: desensitization of response associated with a relatively rapid reduced ability of agonist-occupied receptors to interact with G proteins, and a slower reduction of receptor density. In cultured DRG neurons, two different effects of opioids on $[Ca^{2+}]$i were observed. The large (>25 μm)-sized neurons are more sensitive than small (<16 μm)- and intermediate (16-25 μm)-sized neurons to the opioid-induced increase in $[Ca^{2+}]$i. In contrast, the small- and intermediate-sized neurons are more sensitive than large-sized neurons to the opioid inhibition of the increase in $[Ca^{2+}]$i due to the depolarization by KCl. Substance P is also more frequently detected in small- and intermediate-sized neurons.

REGULATIONS OF INTRACELLULAR FREE CALCIUM IN NEURONAL CELLS BY OPIOIDS

by

Tianlai Tang

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Abbreviations:

AS	antisense oligonucleotide
Bmax	maximum density of binding sites
BNTX	7-benzylidene naltrexone
$[Ca^{2+}]i$	intracellular free calcium concentration
CgTX	ϖ-conotoxin
DADLE	[D-Ala^2, D-Leu^5]enkephalin
DALCE	[D-Ala^2, Leu^5, Cys6]enkephalin
DAMGO	[D-Ala^2, $MePhe^4$, Gly-ol^5]enkephalin
DIP	diprenorphine
DPDPE	[D-$Pen^{2,5}$]enkephalin
DRG	dorsal root ganglion
DSLET	[D-Ser^2, Leu^5]enkephalin-Thr
Fluo-3-AM	Fluo-3 acetoxymethyl ester
Fura-2-AM	Fura-2 acetoxymethyl ester
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
Kd	dissociation constant
ND	neuroblastoma × dorsal root ganglion hybrid cells
NTB	naltriben
NTI	naltrindole-5'-isothiocyanate
U69593	(5a, 7a, 8b)-(-)-N-methyl-N-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]-Dec-8-yl)benzenacetamide
TMB-8	3,4,5-trimethoxybenzoic acid 8-(diethylamino)-octyl ester HCl.

INTRODUCTION

Morphine has been used as an analgesic for a long time. However, little was known about the mechanism of opioid effects until the discovery of opioid receptors and their endogenous ligands around the mid '70s. Since then, many opioid receptors and their subtypes have been identified; their physiological roles have been proposed. To understand the mechanisms underlying opioid effects, especially the devolepment of tolerance and dependence, numerous studies have been focused on every step in the opioid-receptor-activated signaling pathway, including G proteins, adenylyl cyclase, ion channels (*e.g.* Ca^{2+}, K^{+}), protein kinases (*e.g.* PKA, PKC), IP_3, intracellular free Ca^{2+}, and gene expression. However, opioid-induced cellular events have not been fully understood; some of them are even controversial. For instance, activation of opioid receptors has been reported to inhibit calcium channel currents (Mudge et al., 1979; Macdonald and Werz., 1986; Tsunoo et al., 1986; Hescheler et al., 1987; Seward et al., 1991; Schroeder et al., 1991); later evidence suggests that in some circumstances, opioid may enhance calcium currents (Shen and Crain, 1989; 1990) or induce an increase in $[Ca^{2+}]i$ (Jin et al., 1993; 1994). With the advent of fluorescent probes (such as Fura-2 AM and Fluo-3 AM) for the intracellular free calcium, it became possible to detect the real-time changes in $[Ca^{2+}]i$ induced by opioid treatment. A characterization of opioid regulation of $[Ca^{2+}]i$ in cultured neuronal cells is the subject of this dissertation. This study may be helpful in understanding the mechanism of opioid functions such as analgesia, modulation of

neurotransmitter release, and opioid tolerance and dependence.

I. Opioid Receptors and Their Functions

Opioid receptors exist in multiple types. There are three classes of opioid receptors for which associated functions have been well defined. Two of these were originally described in the chronic spinal dog model of Martin et al. (1976). Their finding was based upon the observation that different classes of opiate drugs produced distinct behavioral syndromes and that tolerance to one group of opiates did not result in cross-tolerance to another class of opiate drugs. Martin and his colleagues proposed the existence of three types of opioid receptors: The μ-receptor, so named because of its activation by morphine, mediated analgesia, miosis, bradycardia, and hypothermia. The κ-receptor, activated by ketocyclazocine, appeared responsible for sedation and depression of flexor reflexes, but had no involvement in skin twitch reflex or pulse rate. In addition, another receptor σ, which was activated by SKF 10,047, is now not generally considered to be opioid in nature because the effects of SKF 10,047 and related drugs are not reversed by classical opiate antagonists like naloxone or naltrexone. A third class of receptors described by Lord et al. (1977) on the basis of studies on the isolated mouse vas deferens preparation is activated by the enkephalins, and referred to as δ.

Opioid receptors are widely distributed throughout the neuraxis with particularly dense binding observed in limbic structures, thalamic nuclei, periaqueductal gray, and neural areas which are important for visceral functioning (Mansour et al., 1988).

μ–Opioid receptors are particularly widely distributed in CNS. They are most dense in the neocortex, caudate-putamen, nucleus accumbens, thalamus, hippocampus, amygdala, inferior and superior colliculi, nucleus tractus solitarius, spinal trigeminal nucleus and dorsal horn. A moderate density of μ receptors is observed in the periaqueductal gray and raphe nuclei, while relatively little μ binding is seen in the hypothalamus, preoptic area and globus pallidus. The distribution of opioid receptors corresponds well with their putative roles in pain regulation and sensorimotor integration.

δ-Opioid receptors are more restricted in their distrubution and appear most dense in olfactory tract, neocortex, caudate-putamen, nucleus accumbens, and amygdala. δ–Binding is relatively low in the thalamus, hypothalamus and brainstem. The δ–opioid receptor may play a role in motor integration, olfaction, cognitive functioning, and regulation of pain perception.

κ-Opioid receptors are localized in an intermediate number of brain areas with the densest areas of binding observed in the caudate-putamen, nucleus accumbens, amygdala, hypothalamus, neural lobe of the pituitary, median eminence, and nucleus tractus solitarius. Moderate amounts of κ sites are found in the periaqueductal gray, raphe nuclei, spinal trigeminal nucleus and dorsal horn. The distribution of κ-opioid receptors is consistent with their probable roles in regulating water balance, food intake, pain perception and neuroendocrine function.

Despite a plethora of studies on the physiological and behavioral effects of administering diverse opioid peptides, alkaloids and antagonists, it is still difficult to draw firm

conclusions regarding the exact functional roles of different opioid receptor types in the neuronal system. For example, the μ, δ, and κ opioid receptor types have all been implicated in the mediation of analgesia, but the details of their differential involvement are not clear. Supraspinal mediation of opioid analgesia has frequently been attributed exclusively to μ receptor activation (Fang et al., 1986; Dauge et al., 1987), but later evidence points to the involvement of δ receptors as well (Mathiasen et al., 1987; Porreca et al., 1987) particularly in the medullary reticular formation (Jensen and Yaksh, 1986). At the spinal level, selective μ, δ, and κ agonists are all active in visceral analgesia testing. However, κ–agonists appear to be distinct in that they block mechanical nociceptive responses; this contrasts with the modulation of thermal nociceptive responses by μ and/or δ receptors (Schmauss et al., 1987). These differential actions may be related to differences in the distribution of the multiple opioid receptor types in spinal and dorsal root ganglion sensory neurons.

Based upon the results from binding studies or functional studies, subtypes of μ, δ, and κ receptors have also been identified; the μ class has been further divided into μ_1 and μ_2. μ_1-Receptors have been proposed to mediate analgesia; and μ_2-receptors to mediate respiratory depression (Pasternak and Wood, 1986). These assumptions are based upon the observation that the putative irreversible antagonist, naloxonazine, antagonized analgesia but not the respiratory depression induced by morphine. Naloxonazine is proposed to antagonize actions at μ_1 receptors irreversibly but only shows short lasting reversible antagonism at μ_2 receptors.

Unfortunately, there is to date no antagonist that can reverse the respiratory depression without affecting the analgesia. Nevertheless, if selective μ_1-agonists can be developed, they may be promising agents for the relief of pain with fewer side-effects than non-selective μ-agonists.

Subtypes of κ-receptors have also been proposed (Gouarderes et al., 1986; Zukin et al., 1988). There are two populations of κ-receptors that can be differentiated based on their affinity for benzeneacetamides, such as U50,488 and U69593. κ-Sites sensitive to U69,593-like compounds have been referred to by some investigators as κ_1 and sites having a low affinity for these compounds have been referred to as κ_2 (Clark et al., 1989). The functional significance of these sites remains to be elucidated, although some studies point to a role for these receptors in regulation of neurotransmitter release in the cortex (Kim and Cox, 1994)

Finally, recent studies indicate that opioid δ receptors can be classified into δ_1 (sensitive to DPDPE and DADLE) and δ_2 receptors (activated by deltorphin-II and DSLET) based on the observation that 5'-NTII (δ_2 receptor antagonists) significantly antagonized the antinociceptive effect of deltorphin-II but not DPDPE; in contrast, DALCE (δ_1 receptor antagonist) antagonized the antinociceptive effects of DPDPE but not deltorphin-II (Jiang et al., 1991). In addition, both DPDPE and deltorphin II can cause antinociceptive effects and also tolerance in mice, but there is no cross tolerance developed between these two compounds (Mattia et al., 1991). The molecular basis of differences in δ_1 and δ_2 receptors is not known;

nor is their distribution or specific functional roles. Buzas and Cox (1994) have reported differential regulation of adenylyl cyclase by δ_1 and δ_2 receptors in candate putamen. The δ_1 and δ_2 receptors appear to be located on different striatal neuron populations (Noble and Cox, 1995).

II. G Proteins in Opioid Receptor Meidated Responses

The defining property of G-protein coupled receptors is that the receptor mediated function is dependent on the coincident hydrolysis of guanine triphosphate (GTP); the agonist binding affinity of the receptors is reduced by GTP or other guanine nucleotides. For example, receptor-mediated stimulation or inhibition of adenylyl cyclase requires the presence of GTP. In addition to supporting receptor-mediated adenylyl cyclase activity, GTP (or its nonhydrolyzable analogs) also directly affects receptor function by increasing agonist dissociation rates and thus decreasing agonist affinity. This effect of guanine nucleotides is apparent only with the binding of agonist, since GTP does not affect antagonist binding (Werling et al., 1988). Therefore, it is proposed that the receptor exists in two different affinity states for agonists depending on the presence of guanine nucleotides, while antagonists bind with a single high affinity to both states (Kent et al., 1980).

The effects of guanine nucleotides on signal transduction mechanisms are mediated through specific G-proteins. Stimulation of adenylyl cyclase is mediated by G_s, while inhibition is mediated through G_i. Both proteins are heterotrimers of subunit composition α, β, and γ. Although α_s and α_i are different proteins, they exhibit

considerable similarities. Both subunits bind and hydrolyze GTP and have considerable similarities in primary structure (Manning and Gilman, 1983). The α subunit contains several highly conserved regions which contain the GTP binding site (Itoh et al., 1986), while other regions with much of the variability between subtypes may be responsible for selectivity of coupling with effector systems or with receptors. Other types of G protein, G_o and G_q, have also been identified. G_o, originally found in high concentrations in the brain, is thought to be involved in the functional coupling of δ opioid receptors to calcium channels in the NG108-15 cells (Hescheler et al., 1986,; 1987). G_q may mediate receptor-activated phosphoinositol turnover and activate IP_3-Ca^{2+} signaling pathway.

Cholera toxin and pertussus toxin interact with specific α subunits, by catalyzing ADP-ribosylation of the proteins. The functional consequences of toxin reactions with both G proteins lead to the same result: an increase in adenylyl cyclase activity and an increase in intracellular cAMP. Cholera toxin-mediated ribosylation blocks the αs GTPase responsible for inactivation of adenylyl cyclase (Northup et al, 1983), thus irreversibly stimulating the enzyme. On the other hand, pertussis toxin inactivates the inhibitory function of the $G\alpha_i$ or $G\alpha_o$ subunit, thus stimulating adenylyl cyclase by removing the inhibitory component of the cycle (Katada and Ui, 1982).

Three pieces of evidence indicate that opioid receptors are directly coupled to G proteins. First, GTP is required for opioid inhibition of adenylyl cyclase activity; Second, GTP (or GTP analogues) regulate opioid receptor binding by decreasing binding of

agonists (Werling et al, 1988) without changing antagonist binding. Second, opioid agonists stimulate GTPase activity in cells and membranes which contain opioid receptors (Koski and Klee, 1981). These effects have been observed for μ-, δ- and κ- receptors (Childers et al., 1991).

Among G-proteins, those most clearly shown to be linked to opioid receptors are pertussis toxin-sensitive G proteins (G_i or G_o). Their activation may cause inhibitory effects through diverse cellular events, including inhibition of adenylyl cyclase (δ-receptor: Sharma et al, 1975; μ-receptor: Yu and Sadee, 1986; κ-receptor: Konkoy et al,1988), increasing potassium conductance (μ-receptor in locus ceruleus: North et al., 1987), or to decrease calcium conductance (δ-receptor in NG108-15 cells: Hescheler et al., 1987; μ-receptor in SH-SY5Y cells: Seward et al.,1991; κ-receptor in spinal cord culture: Gross et al., 1990). Since many subtypes of these G proteins, such as $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{o1}$, and $G\alpha_{o2}$ have been identified, purified, and cloned in recent years, it has become possible to study the function of each of these G protein subtypes independently in opioid-induced responses. In NG108-15 cells (which only express δ receptor), there is evidence suggesting that the $G\alpha_{i2}$ subtype is mainly responsible for the inhibition of adenylyl cyclase (Mckenzie and Milligan, 1990). These studies showed that antibodies to $G\alpha_{i2}$, but not antibodies to $G\alpha_{i1}$, $G\alpha_{i3}$, or $G\alpha_o$, inhibited opioid receptor function in NG108-15 cells, including ligand binding, agonist-stimulating GTPase activity, and opioid-inhibition of adenylyl cyclase. In contrast, G_o is suggested to mediate opioid-induced inhibition of Ca^{2+} channels. Intracellular application of G_o

subunits was more effective than G_i in reconstitution of the opioid receptor mediated inhibition of Ca^{2+} current when NG108-15 cells were pretreated with PTX (Hescheler et al.,1988). In the same cells, opiate inhibition of Ca^{2+} current was blocked by preinjecting the cells with antibodies against G_o but not against G_i (Kleuss et al, 1991). Those observations need confirmation in other tissues. Similar assignments of the G protein subtypes to specific effector systems were also made in other hormone-secreting cells, such as GH_3 pituitary cells (Gollasch et al., 1993). In addition to opioid-induced inhibitory effects mediated by PTX-sensitive G proteins, it has been proposed that G_s is involved in opioid receptor-induced stimulatory effects by stimulating adenylyl cyclase activity and calcium influx or by inhibiting potassium conductance (Shen and Crain, 1989; 1990).

III. Regulation of the Cytosolic Calcium by Opioids

There are two basic mechanisms by which the cell may regulate its cytosolic free Ca^{2+}: by controlling entry of extracellular Ca^{2+} through calcium channels and by controlling release of Ca^{2+} from its intracellular stores. Although calcium channels and intracellular calcium stores are operated by different mechanism, they may also influence each other.

1. Calcium channels

Calcium channels may be classified as three types: voltage-operated calcium channels, receptor-operated calcium channels, and second-messenger-operated calcium channels. The best

characterized mechanism of Ca^{2+} influx is through voltage-operated Ca^{2+} channels. Six classes of voltage dependent Ca^{2+} channels (termed L, N, P, T, R, and Q) have been defined on the basis of their physiological and pharmacological properties. The L-type channel, which mediates long-lasting Ca^{2+} currents, is best characterized: it is a dihydropyridine-sensitive channel, and exists as a compex of five subunits: (α_1,α_2, β, γ and δ); The binding site for Ca^{2+} channels blockers is present on the α_1-subunit (Dunlap et al., 1995). A cAMP-dependent protein kinase (PKA) can potentiate Ca^{2+} current, suggesting that L-type channels are regulated by both membrane potential and phosphorylation.

L-type channels are mainly distributed in skeletal muscle, cardiac muscle, smooth muscle, and also in CNS, such as hippocampus, cerebral cortex, cerebellum, spinal cord, and retina (Ahlijanian et al., 1990). They play an important function in the generation of action potentials and signal transduction at the cell membrane. 1,4-Dihydropyridine (DHP) derivatives exhibit antagonistic or agonistic actions on the L-type calcium channel. Bay K 8644 increases the current generated by L-type channel; DHP calcium antagonists, such as nifedipine and nitrendipine, block the L-type channel current.

Another high-threshold calcium channel, mainly found in neuronal cells, is the N-type calcium channel (for neither T nor L). It is insensitive to DHP but blocked by the marine snail toxin, ω-conotoxin (ω-CgTX). It is suggested that N-channels play a dominant role in neurotransmitter release and neurosecretion (Tsien, et al., 1988).

Another recently described high-threshold calcium channel is the P-type calcium channel (named for Purkinje cell). Direct evidence for the existence of P-channels is provided by the observation of a calcium conductance in cerebellum Purkinje cells that is inhibited by funnel-web spider toxin, but not affected by DHP or ω-CgTX (Llinas et al., 1989). P-type calcium channels are widely distributed in mammalian CNS and may participate in neurotransmitter release.

Although initially it was believed that the opening of voltage-operated calcium channels is only under control by changes in the potential across neuronal membranes, later studies showed that some neurotransmitters or hormones may also activate or inhibit voltage-sensitive calcium channels by an action on G protein-coupled receptors.

2. G-protein modulation of the voltage-dependent calcium channels

Many neurotransmitters and neuromodulators are known to regulate calcium-dependent processes in neurons, and a large number of them require the obligatory activation of G protein in the transduction of this chemical signal; the opening-closing state of voltage-sensitive Ca^{2+} channels is strongly influenced by the activation of the specific G protein.

G-protein involved in the opening of Ca^{2+} channels

The first observed G-protein effect in the regulation of Ca^{2+} channels was that of G_s. In cardiac and skeletal muscle cells, G_s- and adenylyl cyclase-stimulating hormones increase dihydropyridine-sensitive (L-type) Ca^{2+} currents by a dual pathway.

Ca^{2+}-channel activity is indirectly enhanced by phosphorylation of channel components by the catalytic subunit of cyclic AMP-dependent protein kinase and, more directly, by a G_s-Ca^{2+}-channel interaction (Trautwein and Hescheler, 1990). G_s protein has also been proposed to be involved in opioid receptor-mediated enhancement of Ca^{2+} influx in sensory neurons (Shen and Crain, 1990).

The pertussis toxin-sensitive G protein G_i, initially identified as an inhibitory regulator of adenylyl cyclase (Yu and Sadee,1986; McKenzie and Milligan, 1990), is also responsible for activation of Ca^{2+} channels in some endocrine cells. In an adrenal cortical cell line (Y-1), angiotensin II stimulates voltage-dependent Ca^{2+} currents (Hescheler et al., 1988). Pretreatment of cells with pertussis toxin abolishes the hormone stimulation of the Ca^{2+} current. In rat pituitary GH_3 cells, thyrotropin-releasing hormone (TRH) stimulates voltage-dependent L-type Ca^{2+} channels. This effect can be blocked by a microinjection into GH_3 cell nuclei of antisense oligonucliotides against G_{i2} but not G_{i1} or G_o α-subunit sequences (Gollasch et al., 1993). Whether the G_i (possibly G_{i2}) mediated activation of voltage dependent Ca^{2+} channel results from the direct interaction between G protein and Ca^{2+} channel or indirectly through other cytosolic components is still under debate. It seems clear that cAMP-dependent phosphorylation is not involved in the G_i protein mediated activation of Ca^{2+} conductance for several reasons. (a) Intracellularly applied cAMP or extracellularly applied forskolin does not stimulate Ca^{2+} currents in Y1 (Hescheler et al. 1988) or GH_3 cells (Rosenthal et al., 1988). (b) In membranes

of adrenocortical and pituitary cells, angiotensin II does not stimulate but rather inhibits adenylyl cyclase (Enjalbert et al., 1986). (c) The fact that the stimulatory Ca^{2+} current is blocked by pertussis toxin indicates that elevation of cAMP is not an intermediate step since stimulation of adenylyl cyclase is believed to be mediated by G_s, a G protein that is a substrate for cholera toxin but not for pertussis toxin. There is a possible involvement of protein kinase C in hormone induced stimulation of Ca^{2+} currents. The studies in GH_3 cells indicate that PKC is also required for TRH-induced stimulation of L-type Ca^{2+} channels based on the observation of inhibitory effects of antisense oligonucleotides directed against $G_q/G_{11}/G_z$ α-subunit sequences and treatment of GH_3 cells with PKC inhibitors, respectively (Gollasch et al., 1993). Therefore, it is likely, that both direct G_i protein-Ca^{2+} channel interaction and G_q/G_{11}-phosphatidylinositol (PI) pathway are independently responsible for some hormone induced activation of Ca^{2+} channels. In addition, there is a report that in rat nodose neurons, calcitonin gene-related peptide (CGRP) enhances N-type Ca^{2+} current via a pertussus toxin-sensitive pathway (Wiley et al., 1992). Whether G_i or G_o is involved in this response is not identified.

G-protein involved in the closing of Ca^{2+} channels

Several neurotransmitter or neuromodulator receptors, including $GABA_B$, $5\text{-}HT_{1a}$, α_2-adrenergic, muscarinic, somatostatin, neuropeptide Y, dopamine D_2, adenosine A_1, and μ-and δ-opiate receptors, have a common feature: their activation induces an

inhibition of Ca^{2+} currents and also an inhibition of adenylyl cyclase activity (Schultz et al., 1985; Jakobs et al., 1985). Inhibition of both Ca^{2+} channels and adenylate cyclase is mediated by pertussis toxin-sensitive G-proteins. Most studies support the suggestion that the G-protein, G_o, is mainly involved in the inhibition of Ca^{2+} current based on the following observation: (a) following the treatment of cells with pertussis toxin, intracellular application of G_o or its α-subunit efficiently restores the ability of agonists to inhibit Ca^{2+} currents in neuroblastoma x glioma hybrid cells (Hescheler et al., 1987, 1988), or rat dorsal root ganglion neurons (Ewald et al., 1988); (b) antibodies against the G_o α-subunit attenuate the inhibitory modulation of Ca^{2+} currents in snail neurons (Harris-Warrick et al., 1988) and in neuroblastoma x glioma hybrid cells (Brown et al., 1989). These observations must be considered in the light of evidence suggesting that G_i is mainly involved in the inhibition of adenylyl cyclase (Gilman et al., 1987).

Although the pertussis toxin-sensitive G-protein mediates both inhibition of Ca^{2+} current and inhibition of adenylyl cyclase, neither a reduction of cytoplasmic cAMP nor the activity of cAMP dependent protein kinase is likely to be involved in the receptor-mediated inhibition of Ca^{2+} currents in neuronal or hormonal cells, because intracellular infusion of cAMP or extracellular application of the adenylyl cyclase-stimulating agent (i.e. forskolin) does not prevent the receptor-mediated inhibition of Ca^{2+} channel activity. These results have been observed for many receptors, including receptors for opioids (Holtz et al., 1986), somatostatin (Lewis et al., 1986), muscarinic (Wanke et al., 1987), and $GABA_B$ receptors

(Dolphin et al., 1989). There is no evidence to support the involvement of phospholipase C in receptor mediated inhibition of voltage-operated Ca^{2+} channels (Schultz et al., 1990). The G-protein mediating the stimulation of phosphoinositide hydrolysis is generally believed to be G_q, a novel G-protein different from the pertussis toxin-sensitive G-protein (G_o or G_i) which appear to regulate Ca^{2+} channels.

In the absence of clear evidence of effects of cytosolic second messengers such as cAMP, cGMP, diacylglycerol and arachidonic acid in G-protein-mediated regulation of voltage-dependent Ca^{2+} channel in neurons and endocrine cells, the underlying mechanism is probably a direct interaction between G protein and Ca^{2+} channels. There are reports on the interaction between G protein activation and the Ca^{2+} channel ligand binding sites. In cultured rat dorsal root ganglion neurons and sympathetic neurons (Dolphin and Scott, 1988), the agonist effect of Bay K-8644 on Ca^{2+} channel currents is promoted by G-protein activation by microinjection of GTPγS into neurons and it is blocked by pertussis toxin. However, there is no strong evidence to demonstrate a direct association between G protein and Ca^{2+} channels.

In conclusion, the pathways through which the interactions of neuronal or hormonal agonists with G protein-coupled receptors cause closing (through G_o) or opening (through G_{i2} or G_q/G_{11}) of calcium channels have been identified. However, the mechanisms by which G proteins actually affect Ca^{2+} channel activity remain unclear; the Ca^{2+} channel subunits or intermediate protein components interacting with activated G protein(s) still need to be

determined by reconstitution, coexpression or antisense oligonucleotide experiments.

3. Opiate regulation of calcium channels

Previous experiments in animal models indicate that Ca^{2+} plays an important role in opioid analgesia. It has been shown that i.c.v. calcium injection antagonizes the analgesic effects of morphine (Kakunaga et al., 1966; Harris et al., 1975; Vocci et al., 1980), β-endorphin and [Met^5]enkephalin (Guerrero et al., 1981). These effects of calcium are potentiated by the calcium ionophores A23187 and X537A (Harris et al., 1975; Vocci et al., 1980; Chapman and Way, 1982). Opiate analgesic effects can be increased by the calcium chelators EDTA and EGTA (Harris et al., 1975; Vocci et al., 1980; Chapman and Way, 1982) and L-type Ca^{2+} channel blockers, such as verapamil, nifedipine, nicardipine and diltiazem (Del Pozo et al, 1987; Contreras et al., 1988). In morphine-tolerant mice, nifedipine has less analgesic effect than in naive mice (Ohnishi et al., 1988). The regulation of Ca^{2+} influx into nerve terminals is likely to be responsible for the effects of Ca^{2+} and Ca^{2+} channel blockers in opioid analgesia.

Direct evidence showing opioid receptor-mediated regulation of Ca^{2+} channel activity comes from electrophysiological studies. There is evidence that all of the opioid receptors, δ, μ, and κ, participate in the regulation of Ca^{2+} currents. μ-Receptor mediated inhibition of voltage-dependent Ca^{2+} currents has been shown in differentiated human neuroblastoma SH-SY5Y cells (Seward et al., 1991) and in rat dorsal root ganglion neurons (Schroeder et al.,

1991; 1993). In both cases, the inhibition of ω-CgTX sensitive N-type Ca^{2+} channel was identified as the responsible mechanism. In guinea pig submucous plexus neurons, activation of δ-opioid receptor results in a decrease in calcium conductance (Surprenant et al., 1990; Shen et al., 1990). Measurement of current through single calcium channels showed that the calcium channels affected were of the N-type (8-12 pS conductance). The observation of the action of [Met^5]enkephalin when applied to the outer surface of excised patches of membrane indicates that no freely diffusible second messenger is required for the transduction between receptor and channel. In neuroblastoma x glioma hybrid cells (NG108-15) expressing δ-receptors, patch-clamp experiments demonstrated an opioid (enkephalin, morphine)-induced block of calcium channels (Tsunoo et al., 1986). In this study, enkephalin inhibited a voltage-dependent Ca^{2+} channel that required large depolarization for activation (referred to as type 2, probably the L or N channels). The original suggestion that κ–agonists inhibit voltage-dependent calcium currents came from observations of the duration of action potentials in mouse cultured dorsal root ganglion cells. Subsequent voltage-clamp recordings show conclusively that the selective κ-agonist, dynorphin, inhibits the N-type calcium current in these neurons (MacDonald and Werz, 1986; Gross and MacDonald, 1987; Gross et al., 1990).

Precisely how activation of opioid receptors results in inhibition of calcium currents is not known. The first step is undoubtedly activation of a G protein. Whole-cell recordings made with electrodes that contain non-hydrolyzable derivatives of GTP

(such as GTPγS) show that the inhibitory action of the opioid becomes irreversible (Brown et al., 1989; Surprenant et al., 1990) as expected when α-subunit is not able to hydrolyze the bound GTP. In other cases, introduction of GTPγS into the cell interior reduces the current directly even in the absence of external opioids (Hescheler et al., 1987; Dolphin and Scott, 1989).

The action of opioids can be entirely blocked by pretreatment with pertussis toxin in all cases in which the experiment has been attempted (NG108-15 cells: Hescheler et al 1987; Brown et al 1989; mouse dorsal root ganglia: Shen and Crain 1989; guinea pig submucous plexus neurons: Surprenant et al. 1990; SH-SY5Y cells: Seward et al 1991). In NG108-15 cells they can also be blocked by preinjecting the cells with antibodies against G_o though not by antibodies against G_i (McFadzean et al. 1989). This result is in agreement with the earlier work of Hescheler et al. (1987), who found that G_o was more effective than G_i in reconstituting the opioid inhibition on Ca^{2+} current in cells in which this had been lost by pretreatment with pertussis toxin. These results support the general notion that G_o is more likely to be involved in the inhibition of Ca^{2+} current.

A pertussis toxin-sensitive G-protein is also involved in the inhibition of adenylyl cyclase by opioid agonists, but most studies suggest that the inhibition of adenylyl cyclase activity is not related to the inhibition of calcium current (Hescheler et al, 1987; Seward et al., 1991). This notion is mainly based upon the observation that the elevation of intracellular cAMP did not prevent opioid inhibition on Ca^{2+} channels (Seward et al., 1991). The effect

of intracellular cAMP on Ca^{2+} channel is somewhat controversial. There are reports that the elevation of intracellular cAMP depressed N-type Ca^{2+} current in both SH-SY5Y and cultured sensory neurons (Seward et al., 1991; Gross et al., 1989). Direct measurement of the intracellular free calcium in NG108-15 cells with fluorescent dye indicated that membrane permeable cAMP analogs (e.g. dibutyryl cAMP), or the elevation of intracellular cAMP by forskolin induced Ca^{2+} influx (Jin et al., 1993; Wang et al., 1993) and enkephalin suppressed this effect (Wang et al., 1993). Another report from the study on rat nodose ganglion neurons shows that application of cAMP-dependent protein kinase in the Ca^{2+} channel recording pipette increased Ca^{2+} current, and made the current more sensitive to the inhibitory action of dynorphin (Gross et al., 1990). It was suggested that the inhibitory effect on Ca^{2+} current by dynorphin was independent of a reduction of the activity of the adenylyl cyclase/cAMP system, and the Ca^{2+} channels phosphorylated by cAMP-dependent protein kinase become more sensitive to the direct inhibition through κ-receptors (Gross et al., 1990).

In single channel studies, the finding that opioids inhibit calcium current in excised membrane patches strongly suggests that a diffusible second messenger (such as cAMP) is not required (Shen et al., 1990). There is now a growing consensus that activated G protein might bind directly to the voltage-dependent calcium channel, and thereby reduce the probability that it opens during membrane depolarization (Brown and Birnbaumer, 1990); reconstitution of the individual protein in artificial membranes or heterologous cells will probably be required to resolve this question.

4. Opiate regulation of the intracellular calcium mobilization

Calcium ions are distributed in intracellular organelles that are able to sequester calcium. These include mitochondria, the endoplasmic reticulum, and the calciosome. The concentration of intracellular calcium may be regulated by release of calcium from calcium stores or uptake into calcium stores. In some neuronal cells and endocrine cells, it has been shown that an internal calcium release is activated by the binding of agonists to cell surface receptors which couple to G-protein (G_q). The intracellular messenger responsible for mediating this effect has been shown to be 1,4,5-inositol triphosphate (IP_3) generated as a result of receptor-dependent activation of phospholipase C. It has been proposed that there is an IP_3 receptor on the surface of the calcium storage oganelles which also acts as calcium channel to control Ca^{2+} release from intracellular stores (Taylor et al., 1992; Penner et al., 1993).

The close relationship between Ca^{2+} channel and intracellular calcium store comes from a number of observations: first, IP_3 or IP_3-generating agonists not only induce calcium release, but also calcium influx. The calcium influx may be due to the modulation of calcium channels by protein kinase C (PKC) (Gollasch, et al., 1993) or directly activated by the release of calcium from intracellular calcium store. Second, the refilling of Ca^{2+} stores depends on the concentration of extracellular Ca^{2+}. Third, ionomycin, a Ca^{2+} ionophore also releases Ca^{2+} from internal stores, suggesting that the influx of calcium may also induce a secondary release of Ca^{2+} from the calcium store.

The mechanism of opiate regulation of the calcium release from intracellular calcium stores remains uncertain. Recently, opioid receptor has been demonstrated to be coupled with the phosphoinositide (PI) cascade in NG108-15 cells (Jin et al., 1994). In this study, opioids activated phospholipase C (PKC), resulting in a subsequent release of Ca^{2+} from an inositol 1, 4, 5-triphosphate (IP_3)-sensitive store. But interestingly, this process was blocked by pertussis toxin, indicating that G_i or G_o protein may be involved. This result needs to be confirmed in other tissues.

IV. Cell Responses to Sustained Exposure of Opioids

Most studies on tolerance to opioid actions mediated by δ-receptors have been conducted in NG108-15 neuroblastoma x glioma hybrid cells. After sustained exposure of the cells to morphine or etorphine, opioids were much less able to inhibit adenylate cyclase (Sharma et al., 1975; Loh et al., 1988). When the very efficacious agonist etorphine was used for chronic treatment, loss of agonist effect occurred within one hour at a time when the number of receptors present was unchanged. A decrease in receptor number was observable at a later time point, indicating that the loss in effectiveness of opioid agonist was a two-step process including both a desensitization and down-regulation.

Most opiate drugs used clinically in humans have selective affinity for μ-type opioid receptors. For this reason, the availability of the 7315c pituitary tumor cell provides an especially useful model for examining the changes in μ-receptor properties and function in a population of cells that bears opioid receptors only of

the μ type. Incubation of these cells with morphine in primary culture resulted in a complete loss of the ability of morphine or other μ-agonists to inhibit adenylate cyclase. Loss of this ability occurred within hours, and again before the reduction in receptor number could be detected. After 24 hours, a loss in μ-receptor number became apparent, and after 72 hours the total μ-receptor density was reduced to about 40% (Puttfarcken et al., 1988). As with δ-receptors in NG108-15 cells, the development of tolerance appeared to involve a two-step process of desensitization followed by down-regulation. In addition to the reduction in receptor number, there was also a loss in the ability of guanyl nucleotides to alter binding affinity for agonists. In membranes prepared from untreated cells, GTP or its non-hydrolysable analogue GTPγS induced an intermediate and a very low affinity state of the μ-receptor. In contrast, the number of high affinity binding sites was much reduced and no very low affinity sites were observed in the presence of GTPγS in membranes prepared from morphine-treated cells. The affinity of radiolabelled μ-agonist for its receptor after chronic morphine exposure was much lower than in membrane from untreated 7315c cells, and was very similar to its affinity after treatment of the 7315c cells with pertussis toxin. Pertussis toxin ADP-ribosylates the G-protein, such that it can no longer interact with the receptor, resulting in a functional uncoupling from the effector system. The similarity of chronic opioid effects and pertussis toxin treatment suggests that tolerance may also involve a functional uncoupling of receptor from effector system.

Compared to the bulk of studies on the long-term actions of

opioids at the receptor and adenylyl cyclase, there is less information relating to opioid regulation of ion channel functions. Crain et al. (1988) observed that mouse sensory dorsal-root ganglion (DRG) neurons chronically exposed to 1 μM D-Ala^2-D-Leu^5-enkephalin (DADLE) for more than one week in culture become tolerant to the inhibitory effects of opioids, i.e. shortening of the duration of the calcium-dependent component of the action potential duration (APD). Acute application of higher concentration of DADLE (10 μM) to these treated neurons not only fails to shorten the APD but, instead, generally elicits excitatory effects, i.e. prolongation of the APD. This action potential broadening effect is also found in nontolerant cultures, but at much lower agonist concentrations than required to shorten the action potential duration (Shen and Crain 1989; Crain and Shen 1990). DADLE- or morphine-treated DRG neurons also become supersensitive to the excitatory effects of opioids. Whereas 1-10 nM dynorphin (1-13) are generally required to prolong the APD of naive DRG neurons, concentrations as low as 1-10 fM became effective after chronic opioid treatment. It is quite a challenge to provide a mechanism for these results. Based on the observation that acute GM1 ganglioside treatment of DRG neurons mimics the supersensitivity to the excitatory effects of opioid agonists under chronic opioid treatment, Crain et al (1990) proposed that opioid excitatory supersensitivity in chronic opioid-treated DRG neurons may be due to cyclic AMP-dependent increase in GM1 ganglioside levels, which may allosterically regulate Gs-coupled excitatory opioid receptor function. Direct evidence in supporting this hypothesis is lacking, and the physiological significance of the

observation is not very clear. The authors suggested that the supersensitivity to excitatory effect of low concentration of opioid under chronic opioid treatment may account for some of the hyperexcitable and hyperalgesic behavior observed after withdrawal of opioids (Crain et al., 1992).

A group of studies on possible changes in the density of L-type Ca^{2+} channels after opioid treatment show that acute treatment of hippocampal slices with morphine followed by the preparation of membrane fractions reveals the presence of low affinity binding sites for [^{3}H]nitrendipine without a change in the total number of binding sites; while prolonged administration of morphine increases the number of [^{3}H]nitrendipine binding sites (Ohnishi et al 1989,1991). These results suggest that an increase of L-type Ca^{2+} channel density may be one of the reasons that chronic treatment with opioid induces insensitivity to opioid inhibitory actions on Ca^{2+} influx. However, direct evidence from Ca^{2+} channel recording under chronic opioid treatment is still missing.

V. Excitatory Effects of Opioid

Although many effects of opioids are inhibitory, recent evidence suggests that under some circumstances opioid receptors may induce excitatory effects. Makman et al. (1988) have reported that in primary cultures of fetal mouse spinal cord-dorsal root ganglia, the basal activity of adenylate cyclase (i.e. in the absence of forskolin or other added stimulants) was increased by 2 μM levorphanol. Naloxone (2 μM) also stimulated adenylate cyclase activity, and antagonism of the stimulatory effect of levorphanol by

naloxone was not demonstrated. When the enzyme was activated by forskolin, the anticipated inhibitory effect of levorphanol was observed, and this inhibitory effect was naloxone reversible. Chronic treatment of the cultures with morphine resulted in an enhancement of the stimulatory effects of levorphanol and a reduction of its inhibitory effects on adenylyl cyclase. Opioid receptor-mediated stimulation of adenylate cyclase activity has also been observed in rat olfactory bulb (Onili et al., 1991; Olianas et al., 1993). In this system, δ and μ receptor agonists stimulate adenylate cyclase activity dose-dependently. As this effect can be blocked by pertussis toxin, therefore G_o/G_i protein is suggested to be involved in this response (Onili and Olianas, 1991).

With respect to ion channels, Crain and his colleagues have observed that opioids induced an increase in the duration of calcium-dependent action potentials in dorsal root ganglion neurons in cultures. Low concentrations (1-10 nM) of opioids with μ-,δ-, and κ-receptor selectivity are all reported to increase action potential duration in about two-thirds of neurons tested, while high concentrations (around 1 μM) of the same agonist reduced action potential duration (Shen and Crain 1989). Pertussis toxin treatment of the cultures enhanced the stimulatory effects and reduced or abolished the inhibitory effects of the opioids. A role for cAMP in the stimulatory effects of opioids suggested by the observation that the opioid-induced prolongation of the action potential was blocked by an inhibitor of cAMP-dependent protein kinase (Chen et al., 1988), by treatment with cholera toxin, which is known to activate G_s and therefore might prevent further activation by opioids (Shen and

Crain, 1990). These results suggest that in this preparation low concentrations of opioids activate G_s, resulting in an increase in adenylate cyclase activity and activation of cAMP-dependent protein kinase, which may further modify ion channel function to cause an increase in action potential duration. In a neuroblastoma x DRG neuron hybrid cell line F11, It has been demonstrated that μ and δ opioid agonists at low concentration decreased voltage-dependent K^+ currents via cholera toxin-sensitive receptors (Fan et al. 1993).

Elevations of intracellular calcium after treatment with δ agonists have also been demonstrated in NG108-15 cells (Jin et al, 1992; 1994). In differentiated cells, δ-receptor agonist DADLE mediated the Ca^{2+} influx through dihydropyridine-sensitive Ca^{2+} channels. This effect is suggested to be due to membrane depolarization as it can be inhibited by the Na^+ channel blocker, tetrodotoxin. In undifferentiated cells, the DADLE-induced increase in $[Ca^{2+}]i$ has been possibly augmented by Ca^{2+} release from IP_3-sensitive store (Jin et al., 1994). The $[Ca^{2+}]i$ increase can also be mimicked by both dibutyryl cAMP and phorbol 12,13-dibutyrate, which lead the author to suggest that cAMP and protein kinase C (PKC) may mediate the increase in $[Ca^{2+}]i$ induced by opioid.

In spite of the general agreement that the cytosolic second messengers (e.g. cAMP, IP_3, DG) are not required for the opioid inhibitory effect either through closing Ca^{2+} channels or opening K^+ channels, the role of second messenger in opioid stimulatory effect through activating Ca^{2+} entry or blocking K^+ current is not very clear. Considering the reports that opioids stimulate adenylate cyclase, and protein kinase C (Chen et al., 1991) in certain tissues,

it is possible that protein kinase A or C modifies Ca^{2+} channels (opening) or K^+ channels (closing), and therefore stimulate neurons. However, a direct interaction between G-protein and Ca^{2+}/K^+ channel is still possible.

There are other reports suggesting that low concentrations of opioids may exert stimulatory effects in different assay systems. Gintzler and Xu (1991) report that low concentrations (1-5 nM) of μ-, δ-, and κ-selective agonists enhance electrically stimulated release of [Met^5]enkephalin from superfused guinea pig myenteric plexus preparations, and that this effect is blocked by cholera toxin treatment. Higher concentrations of the same opioids (100 nM and 1 μM) inhibited release of the peptide. The inhibitory action was unaffected by cholera toxin but inhibited by prior pertussis toxin treatment. Opioids have also been reported to induce the release of adenosine in spinal cord (Sawynok et al., 1989; Sweeney et al., 1989) and of substance P from trigeminal nucleus (Suarez-Roca and Maixner, 1993); the physiological significance remains unclear. In addition, there is a proposal that opioids may participate in the facilitation of long-term potentiation through an increase in $[Ca^{2+}]i$ and other second messengers (Bramham et al., 1988; Huang, 1992).

V. Summary and Objectives

In regard to opioid induced cellular events in neuronal tissues, it has been known that opioids inhibit adenylyl cyclase activity through $G\alpha_{i2}$ coupled receptors, and N-type calcium channels via $G\alpha_o$. The inhibitory effects of opioids may also be induced by the opening of K^+ channels. On the other hand, it has been also proposed that

opioids enhance voltage-sensitive calcium currents and induce the mobilization of Ca^{2+} from intracellular stores in certain tissues. Opioids may also activate adenylyl cyclase activity in some circumstances. However, the specific G proteins and the physiological significance involved in these opioid-induced stimulatory actions are not yet established. In regard to the cellular model for opioid-induced tolerance and dependence, it has been revealed that chronic treatment of tissue or cultured cells with opioid agonist induces a desensitization of opiate inhibition of adenylyl cyclase activity. This process is associated with a relatively rapid reduction of receptor affinity for agonist and a slower down-regulation of receptor number. In some circumstances an increased activity of adenylyl cyclase is also observed. The effects of chronic opiate treatment on Ca^{2+} channels, especially on the regulation of intracellular free calicum by opioids are still not fully understood.

The regulation of $[Ca^{2+}]i$ by opioids in sensory neurons is regarded as one of the main mechanisms involved in opioid analgesic effect, in the modulation of neurotransmitter release by opioids, and perhaps in opioid tolerance mechanisms. With the advent of the fluorescent probe-based fluocytometry, it is now possible to observe real time changes in the $[Ca^{2+}]i$ in living single cell; thus we can obtain information about the kinetics of the $[Ca^{2+}]i$ influenced by drugs and also information about potential diverse responses occurring in sub-populations of cultured cells. Our objective was to explore how opioids regulate $[Ca^{2+}]i$ in sensory neurons. In the first part of this project, I characterized effects of

acute and prolonged treatments with opioids on $[Ca^{2+}]i$ in a cultured DRG cell line, ND8-47; I then analyzed the mechanism(s) involved in these effects in the following main aspects: calcium channels, intracellular calcium stores, G protein, opioid binding states in both acute or chronic opioid treatment. In the second part, I examined opioid effects on $[Ca^{2+}]i$ in cultured fetal mouse dorsal root ganglion neurons, and the response in the sub-population of the larger-sized neurons resembled that observed in ND8-47 cells.

METHODS

Culture of ND8-47 cells

ND8-47 cells were grown either in 35 mm culture dishes (Costar, Cambridge, MA 02139) or on glass coverslips (9 × 35 mm, Clay Adams, Lincoln Park, NJ), and maintained in 35 mm culture dishes in a 5% CO2 incubator at 37°C. Cells were cultured in L-15 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO). Cell differentiation was induced by adding 1 mM dibutyryl cAMP (Sigma Chemical Co., St. Louis, MO) and 20 ng/ml nerve growth factor (Sigma Chemical Co., St. Louis, MO) into L-15 medium containing 0.5% fetal bovine serum and continuing culture for 4-5 days.

Culture of dorsal root ganglion neurons

Spinal cord attached with DRGs were taken from 12- to 14-day old mouse (C57BL6J) embryos. DRGs were separated from the cord by removing the meninges with attached DRGs. Dissections were performed in sterile D1 solution consisting 50 ml of 20x concentrated Puck's D1 salt solution (Colorado Serum); 6 g/l glucose; 10 g/l sucrose; 10 mM HEPES (free acid) buffer; osmolality was adjusted to 320-330 mmol/kg by adding sucrose and pH was adjusted to 7.3-7.4 by adding NaOH. After treatment with 0.125% trypsin for 30 min at 35°C, DRG cells were plated into vitrogen-coated 35 mm culture dishes at a density of 0.5 million cells/dish. Neurons were cultured in N3 medium composed of minimum essential medium (MEM) containing 5 % horse serum, 200 μg/ml transferrin, 200 μM putrescine, 60 nM sodium selenite, 20 ng/ml corticosterone,

40 nM progesterone, 20 ng/ml triiodothyronine, 10 μg/ml insulin, 2 mM glutamate and 25 ng/ml nerve growth factor (Sigma Chemical Co., St. Louis, MO). Mitosis of non-neuronal cells was inhibited by adding 5'-fluoro-2'-deoxyuridine (FdU) plus uridine (final concentrations of 13 μg/ml and 33 μg/ml, respectively) to the medium for 2-3 days starting 2 days after plating. Subsequent medium changes were made every three days using N3 medium alone. Cultures were maintained in 10% CO_2 at 35°C.

Procedures for chronic treatments of cells with PTX, CT or opioid peptides

For treatment with pertussis toxin or cholera toxin (Sigma Chemical Co. St. Louis, MO), cells were incubated with each toxin for 24 h in culture medium. $[Ca^{2+}]i$ was measured in Hanks' solution with same concentration of pertussis toxin or cholera toxin. For opioid chronic treatment, cells was incubated with DSLET (Cambridge Research Biochemical Co., Wilmington, DE) for 0.5-24 h, or DPDPE (Cambridge Research Biochemical Co., Wilmington, DE) for 24 h in culture medium. Medium was replaced with fresh medium containing the same concentration of peptide after 12 h treatment. After incubation with opioids, the cells were washed 5 times with Hanks' solution at 37°C to remove residual opioids before the measurement of $[Ca^{2+}]i$.

Preparation of ND8-47 membranes for binding assay

ND8-47 cells were grown in 175 cm^2 tissue culture flasks in either normal medium (L-15 medium containing 10% fetal bovine

serum, 2 mM glutamine, NaHCO2, and glucose) or differentiating medium. Cells were collected by discarding the medium and rinsing the flasks with Krebs buffer. The cell suspension was centrifuged at 450 × *g* for 5 min. The pellet was homogenized in ice cold Krebs-HEPES buffer, using a Teflon-glass homogenizer (Tekmar Company, Cincinnati, OH) at a setting of 8 for 10 sec. The supernatant was then centrifuged at 1000 × *g* for 15 min. The supernatant was centrifuged again at 15,000 × *g* for 30 min. The pellet (cell membrane) was resuspended in ice cold Krebs HEPES buffer and stored frozen in -70°C.

Opioid binding assays

Binding of [^{3}H]Diprenorphine (Amersham, Arlington Heights, IL) to ND8-47 cell membrane was estimated essentially as described previously (Werling et al., 1988). Briefly, ND8-47 cell membrane suspensions were thawed at room temperature. Triplicate samples of membrane suspension in Krebs-HEPES buffer, PH 7.4 were preincubated for 5 min at 37°C with or without nonradioactive displacer. Radiolabeled ligand was then added and the incubation was continued for 20 min. Each sample was then filtered through GF/B glass fiber filters and the filters were rinsed with ice cold Krebs-HEPES buffer 3 times and transferred into liquid scintillation vials for counting. Saturation and displacement data were analyzed by a non-linear curve fitting program, LIGAND (Munson & Rodbard, 1980). Considering that opioid receptors exist in two different binding affinity states for agonists depending on the fraction of receptors that couple to G protein, we used both the one-binding site model

and the two-binding site model to analyze the opioid agonists binding results.

Nitrendipine binding assays

The measurements of binding of [^{3}H]nitrendipine (DuPont Medical Prod. Wilmington, DE) to rat brain tissues treated with morphine were previously reported by Ohnishi et al (1989, 1991). We adapted their method for [^{3}H]nitrendipine binding in ND8-47 cell membranes. ND8-47 cell membrane suspensions in 50 mM Tris-HCl buffer were preincubated for 5 min at 37°C with or without nonradioactive nitrendipine (Calbiochem, Orange, CA) (0.01-300 nM). Radiolabeled nitrendipine (1 nM) was then added and the incubation was continued for 30 min. Non-specific binding was measured by co-incubating 1 nM [^{3}H]nitrendipine with 2 μM unlabeled nitrendipine. The reaction was terminated by filtering the incubation medium through GF/B glass fiber filters. The filters were then washed three times with 5 ml of ice-cold incubation medium and the radioactivity was counted in a liquid scintillation counter.

Intracellular calcium concentration measurements in ND8-47 cells

Fura-2 studies

Confluent monolayers of cells grown on coverslips were incubated with 5 μM Fura-2-AM (Molecular Probes, Inc., Eugene, OR) plus 0.2% pluronic F-127 (to make cells more permeable to Fura-2-AM) in Na^+ Hanks' solution (NaCl, 145 mM; KCl, 4.5 mM; $MgCl_2$, 1.3 mM; $CaCl_2$, 1.6; and HEPES, 10mM) containing 5 mM glucose and 0.2% bovine serum albumin, for 60 min at 37°C. Cells were then washed twice with Na^+ Hanks' solution before fluorescence measurements.

Cells on coverslips were placed in a cuvette containing 2 ml of Na^+ Hanks' solution with temperature regulated by a thermostat (37°C). The fluorescence signal was measured with emission at 510 nm and dual excitation at 340 nm and 380 nm (slit width 4 nm) in a PTI Delta Scan Spectrofluorometer (Photon Technology International, South Brunswick, NJ). Determinations of $[Ca^{2+}]i$ were calculated according to the following formula (Grynkiewicz et al., 1985): $[Ca^{2+}]i = K_d(R - R_{min})(Sf)/(R_{max} - R)(S_b)$, where R is the ratio of fluorescence intensity at 340 nM and 380 nM; R_{min} and R_{max} are the ratios at zero calcium and saturating calcium, respectively; Kd is the dissociation constant for Fura-2; S_f and S_b are the fluorescence intensities of the dye measured at 380 nm in the absence of Ca^{2+} and with saturating Ca^{2+}, respectively. Agents were added to the cuvette in 20 µl aliquot.

Fluo-3 studies

Cells grown in 35 mm culture dishes were loaded with 5 µM Fluo-3-AM (Molecular Probes, Inc., Eugene, OR) plus 0.05% pluronic F-127 for 60 min at 37°C, and then rinsed twice with Na^+ Hanks' solution (cells were still adhering to the bottom of the culture dish). Agents in 10 µl aliquot were added to the dishes containing 1 ml of Hanks' solution. Fluorescence was measured with an ACAS 570 Argon laser cytometer (Meridian Instrument Co., Okemos, MI). The laser excitation wavelength was 488 nm and the emission wavelength was 525 nm.

Measurement of intracellular calcium in DRG neurons

Cells used for experiments were cultured for 12 to 18 days in

35 mm culture dishes. Cells were loaded with 5 μM Fluo-3-AM in D1 solution for 60 min at 35°C, and then rinsed twice with D1 solution (cells were still adhering to the bottom of the culture dish). Fluorescence image was recorded using an ACAS 570 Argon laser cytometer (Meridian Instrument Co.) with setting the excitation wavelength on 488 nm and the emission wavelength on 525 nm. Cells were treated by adding each agent in 10 ml aliquot to the dish containing 1 ml of D1 solution with 1.6 mM calcium. Considering the existence of background noise, we defined a significant change of intracellular calcium level as a change of fluorescence intensity more than 100 unit (corresponding to 20 nM of $[Ca^{2+}]i$). The $[Ca^{2+}]i$ was calculated from the fluorescence measurement using the equation: $[Ca^{2+}]i = K_d(F-F_{min}) / (F_{max}-F)$ where F is the observed fluorescence using Fluo-3 and the value for K_d is 400 nM (Kao et al, 1989). F_{max} was established in cells using 5 mM of the ionophore ionomycin; to obtain the F_{min} value, the Fluo-3-Ca^{2+} complex was eliminated in the same cells by adding 1 mM EGTA and 5 mM Mg^{2+}.

Oligonucleotide treatment of cells

The strategy for designing the 21-26 mer phosphorothioate oligodeoxynucleotides was adapted from ref. Gollasch et al., 1993. They were synthesized using a DNA synthesizer (Applied Biosystems 392) and purified by reverse-phase high-performance liquid chromatography. Each of the oligomers has phosphorothioate groups on the four nucleotides at 5' and 3' end. The oligomers had the following sequences: α_{i2}-antisense (AS), 5' CGGCAGCACAGGACAGTGC GAACAGC 3', corresponding to nt 317-342 of the identical strand of

the $G\alpha_{i2}$ gene sequence; α_{i3}-antisense (AS), 5' CAGCACTGCCAGC TAAAACAA 3', corresponding to nt 322-342 of the identical strand of the α_{i3} gene sequence; α_{i2} sense: 5' GCTGTTCGCACTGTCCTGTGCTG CCG 3'; α_s-antisense (AS), 5' GCACCAGGTTGCTCATGGCGG 3'.

ND8-47 cells were cultured in flasks or dishes containing regular L-15 medium with 10% fetal bovine serum until confluence. The regular L-15 medium was replaced by differentiating medium and oligonucleotides were added into the medium as 100 x stock solutions to give a final concentration of 10 μM. Medium was removed and replaced with fresh medium containing 10 μM oligonucleotides every 48 hours.

Gel electrophoresis and immunoblotting

ND8-47 cell membrane proteins (approximately 20 μg) were run on a 12% SDS-polyacrylamine gel under 125 volts for 4 Hr. The proteins were then transferred from gel to nitrocellulose membrane (for 1 hr at 100 volts). The nitrocellulose membranes were cut into strips containing each lane and placed in strip trays. After incubation in blocking buffer (100 mM Tris-HCl, 0.9% NaCl, 0.1% Tween 20, 5% non-fat milk, pH 7.5), strips of the nitrocellulose were incubated with the following antisera provided by Dr. Allen Spiegel (NIH): QL (selective for α_q) at 1:500; AS (selective for α_{i1} and α_i2) at 1:4,000; GC (selective for α_o) at 1:1,000; EC (selective for α_{i3}) at 1:1,000; and RM (selective for α_s) at 1:4,000. These antisera were successfully used in detecting Ga subunits in other tissues (Goldsmith et al, 1987, 1988; Shenker et al, 1991). The location of each primary antibody was detected with a Vectastain

ABC-alkaline phosphatase kit (Vector Laboratories, Burlingame, CA). The presence of alkaline phosphatase was determined with a Vector alkaline phosphatase substrate kit II (Vector Laboratories, Burlingame, CA).

Immunocytochemistry for Substance P

Cultures of DRG neurons were fixed with 4% paraformaldehyde in 0.1M phosphate buffer (PBS) for 4 h at 4°C, followed by rinses with PBS three times for 10 min each. The cells were then incubated with rabbit antiserum (dilution 1:500) directed against Substance P (INCSTAR, Stillwater, MN) overnight at room temperature. The following day, the antiserum solution was removed and the cultures rinsed three times (5 min for each rinse) with PBS. The cells were subsequently incubated for 1 h in room temperature with a donkey-rabbit IgG antibody coupled to peroxidase. After rinses for three times (5 min for each rinse), peroxidase activity was detected by diaminobenzidine substrate kit (Vector laboratories). We also used 10 μM substance P prior-incubating substance P antibody (4°C, overnight) as a control.

Statistical Analysis

The statistic used in comparison of $[Ca^{2+}]i$ in the presence of opioids to resting $[Ca^{2+}]i$ was Student's *t* test; the data are represented as mean ± S.E.M. For opioid receptor binding assay, the value of K_i and B_{max} are represented as mean (%CV); %CV is a percentage coefficient of variation (standard error divided by the parameter value). The goodness of fit for a two-site model is compaired to a one-site model by F-test.

RESULTS

Section I
Opioid Receptors and Opioid Induced Increase in $[Ca^{2+}]i$ in ND8-47 Cells

Neuronal cell lines are widely used in studes examining the properties of neurons because of the homogenous morphology and specific properties of the cell lines, and because it is possible to obtain adequate number of cells for the biochemical studies (e. g. receptor binding assay, measurement of second messenger levels). In the begining of our studies, I screened a total of 16 ND cell lines (N18Tg2 mouse neuroblastoma x rat DRG neuron hybrid cells, developed by Wood et al.,1990) for expression of opioid receptors. One of these subclones, ND8-47, has high density of opioid binding sites. Since ND cells have been reported to express some of the properties of nociceptive sensory neurons (Wood, et al., 1990; Dunn et al., 1991), the ND8-47 cell line might be used as a cellular model to study opioid actions on sensory neurons.

1. Opioid binding studies

The non-selective antagonist, [^{3}H]Diprenorphine (DIP), has been used to screen 16 subclones of the ND cell line for opioid receptors. Specific binding of DIP by each cell line is reported in Table 1. The ND8, ND8-34, and especially the ND8-47 cell lines showed the highest levels of specific binding of DIP. Induction of cell differentiation by adding dibutyryl cAMP and nerve growth factor in the medium did not significantly alter the binding of [^{3}H]DIP (Table 1). In view of the relatively high density of specific binding sites

TABLE 1

Cell lines	Specific [^{3}H]DIP binding(dpm ± range/mg protein)	Specific binding as % of total binding
ND 11	571 ± 125	18%
ND 7-23	623 ± 89	24%
ND D	847 ± 203	27%
ND 20	911 ± 111	8%
ND 27	2200 ± 762	12%
ND 26	2820 ± 284	9%
ND 5	3570 ± 673	33%
ND 15	4130 ± 376	41%
ND 12	4230 ± 806	42%
ND C	4650 ± 491	47%
ND 7	4790 ± 1180	41%
ND 21	4880 ± 505	29%
ND 22	5960 ± 481 (* 5270 ± 981)	46%(*44%)
ND 8-34	9650 ± 296 (*10500 ±1870)	79%(*83%)
ND 8	20000 ± 2900 (*17800 ±3380)	83%(*86%)
ND 8-47	37200 ± 4070 (*33300 ±2610)	85%(*91%)

Specific opioid binding to neuroblastoma × dorsal root ganglion neuron hybrid cell lines.

Specific [^{3}H]diprenorphine (DIP) binding was calculated by subtracting the non-specific binding from the total binding. Total binding was measured after incubation of the cells membrane with [^{3}H] diprenorphine (1 nM) for 30 min at 37°C, followed by rapid filtering and washing with cold buffer. Non-specific binding was measured by co-incubating 1 nM [^{3}H]DlP with 2 μM unlabeled diprenorphine. Cells were cultured in undifferentiated medium or *differentiated medium. Results are mean values ± range from triplicate determinations in a single assay, except for values for ND8-47, ND8 and ND8-34 cell lines which are means values ± range from triplicate determinations in two independent experiments.

for DIP in the ND8-47 cells, we chose this cell line for further opioid receptor binding studies. The [^{3}H]DIP binding sites on the ND8-47 cell membranes were characterized by measuring the binding of [^{3}H]DIP to homogenate of ND8-47 cell membranes in the presence of DPDPE and DSLET (δ), DAMGO (μ), or U69593 (κ). Fig. 1 shows that deltorphin-II, DSLET and DPDPE all have similar inhibitory activity on the [^{3}H]DIP binding; however, both DAMGO and U69593 showed no inhibition. It is clear, therefore, that the [^{3}H]DIP binding sites in ND8-47 cells are δ-opioid binding sites. [^{3}H]DIP saturation binding experiments were conducted to estimate the total number of binding sites. Computer fitting of the [^{3}H]DIP saturation curve in membranes to a one site model gave a Bmax value of approximately 200,000 sites/cell (Fig. 2).

2. Acute effect of opioid on intracellular free calcium

Fura-2 study

The resting [Ca^{2+}]i in ND8-47 cells differentiated in L-15 medium with dibutyryl cAMP and nerve growth factor was 145 ± 2 nM (total n = 45, from 16 separate experiments). To test opioid effects on [Ca^{2+}]i, the specific opioid receptor agonists DSLET (δ), DPDPE (δ), deltorphin-II (δ), DAMGO (μ), and U69593 (κ) were used in concentrations ranging from 1 nM to 10 μM. Considering the δ-type receptor may further be divided into δ_1 and δ_2 subtypes, DPDPE and deltorphin-II were chosen because of their relative selectivity for these receptor subtypes, respectively (Sofuoglu et al., 1993). A typical trace curve for the increase in [Ca^{2+}]i induced by DSLET is shown in Fig. 3. Upon adding DSLET at 10 nM, 100 nM, and 1 μM,

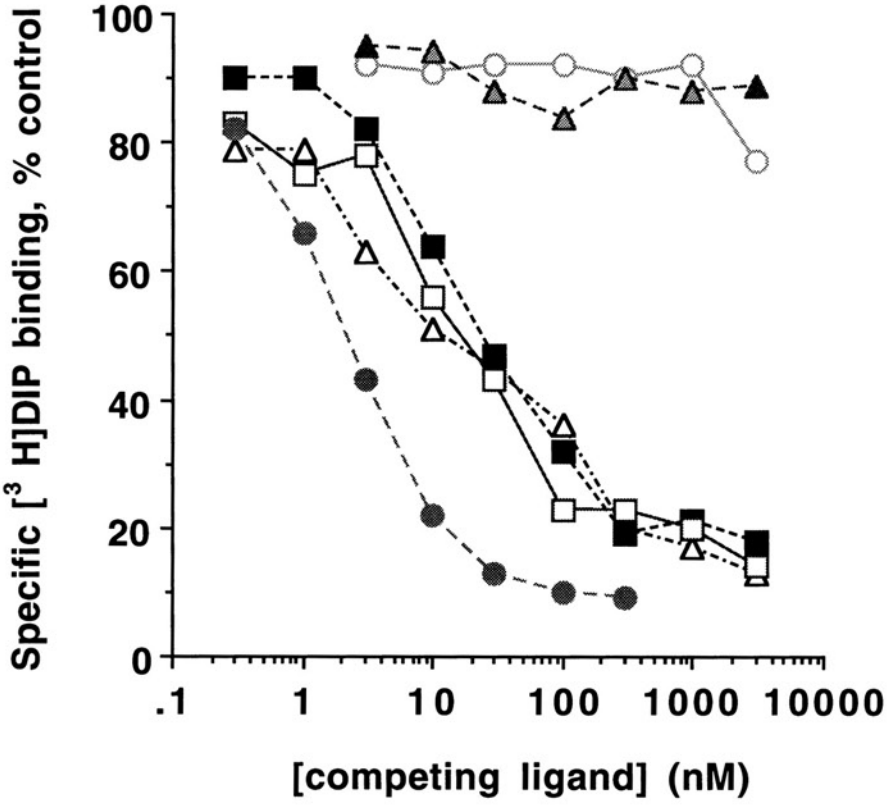


Fig. 1 Competition by opioid ligands against [^{3}H]DIP binding. ND8-47 cell membrane homogenates were incubated with increasing concentrations of diprenorphin (DIP) (●), DPDPE (Δ), DSLET (■), deltorphin-II (□), DAMGO (O), or U69593 (▲) and 1 nM [^{3}H](DIP) for 30 min at 37°C, followed by rapid filtering and washing with cold buffer. Non-specific binding was measured by co-incubating 1 nM [^{3}H]DIP with 2 μM unlabeled DIP. Values represent means from three experiments, each performed in triplicate.

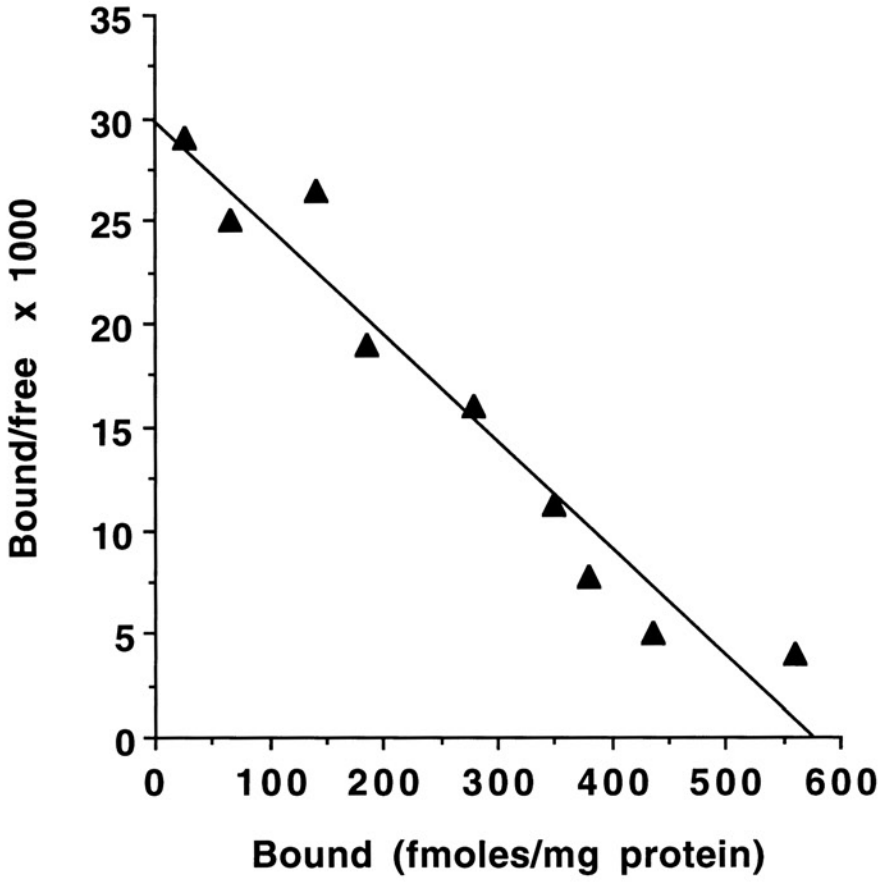


Fig. 2. Scatchard analysis of [^{3}H]DIP binding to ND8-47 cell membranes.

Cell membranes were incubated with [^{3}H]DIP for 30 min at 37°C in the concentration range of 0.1 to 30 nM, and specific binding was assayed. Nonspecific binding was measured in the presence of 30 μM DIP. Bmax = 572 fmole/mg protein; Kd = 1.05 nM. Bmax and Kd values were estimated from the pooled data by non-linear regression (LIGAND) from three experiments.

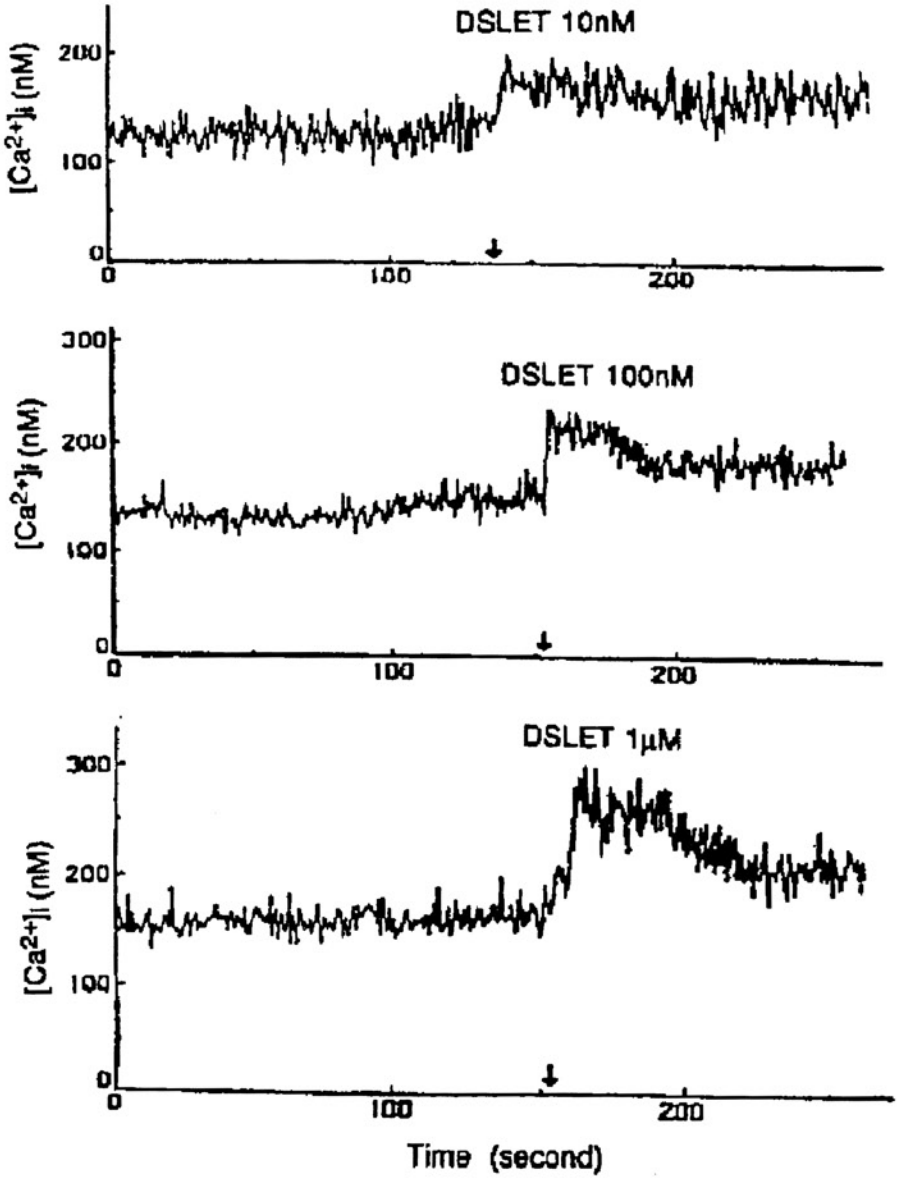


Fig. 3. Fura-2 tracing of $[Ca^{2+}]i$ in ND8-47 cells.
Cells were exposed to DSLET (0.01 μM, 0.1 μM, and 1 μM). Tracings shown are from a typical experiment repeated three to six times with similar results.

$[Ca^{2+}]i$ elevated, reached the peak level within 20-30 sec and then returned to baseline gradually. Therefore, in subsequent studies with Fura-2, opiate effect on $[Ca^{2+}]i$ was measured between 20 and 30 sec after adding opioid to the fluorescent cuvette. The δ-receptor agonists, DSLET, and deltorphin-II induced a substantial increase in $[Ca^{2+}]i$ in dose-dependent fashion (Fig. 4). Unlike DSLET and deltorphin-II, DPDPE only induced a slight increase in $[Ca^{2+}]i$. The μ-selective agonist DAMGO and the κ- selective agonist U69593 only weakly increased $[Ca^{2+}]i$, even at high concentrations (1 μM or above).

To determine whether the increase in $[Ca^{2+}]i$ required activation of opioid receptors, naloxone (a non-selective opioid-receptor antagonist) and naltrindole (a δ-receptor specific antagonist) were used to evaluate their blocking effects on the increase in $[Ca^{2+}]i$ induced by DSLET (100 nM) (Fig. 5). Both naloxone and naltrindole blocked the DSLET-induced increase in $[Ca^{2+}]i$ with the IC50s of 20 nM and 2.5 nM respectively. The greater potency of naltrindole supports the view that the increase in $[Ca^{2+}]i$ induced by DSLET in ND8-47 cells is a δ-type opioid receptor-mediated process. Considering the opioid δ receptors being further divided into δ_1 and δ_2 sub-types, the sub-type specific δ-receptor antagonists, BNTX (δ_1) and NTB (δ_2) (Sofuoglu et al. 1991; 1993) were used to characterize further the type of δ-receptors involved in this response. Fig. 6 shows that NTB was substantially more potent than BNTX in antagonizing the DSLET (100 nM)-induced increase in $[Ca^{2+}]i$. High concentrations of BNTX failed to inhibit completely the increase in $[Ca^{2+}]i$ induced by DSLET. These results, and the much

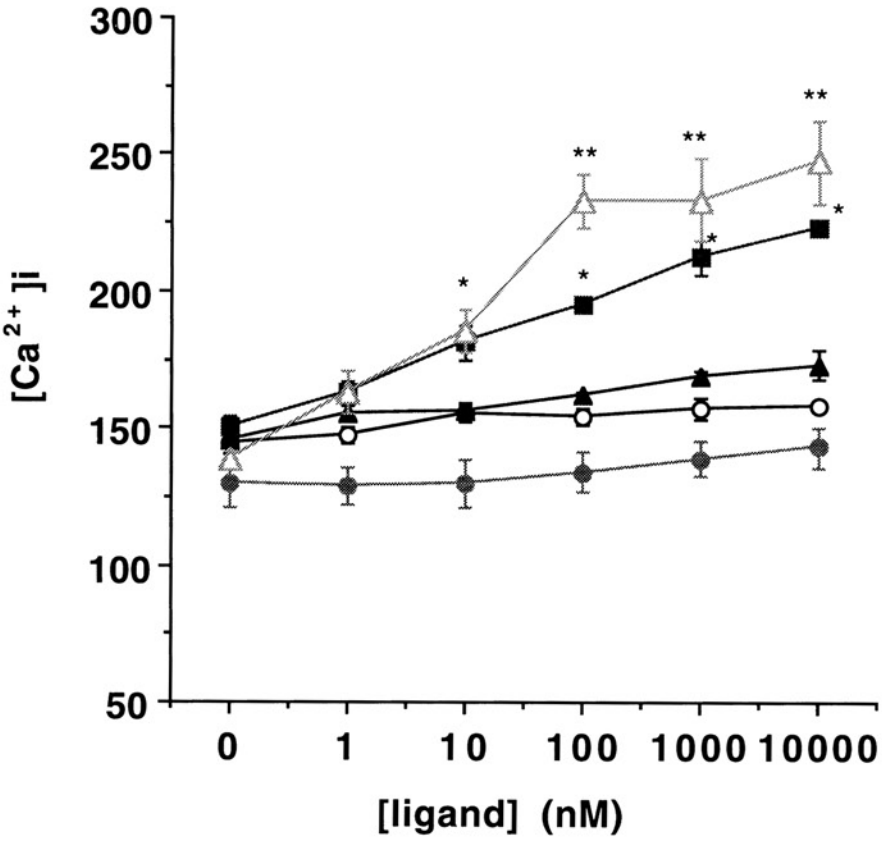


Fig. 4. Opioid effects on $[Ca^{2+}]i$ in ND8-47 cells.
The cells were tested with three δ-receptor ligands: DSLET (△), deltorphin-II (■), and DPDPE (▲); μ-receptor ligand: DAMGO (○); and κ-receptor ligand: U69593 (●) with the concentrations from 1 nM to 10 μM. Values are Means ± S. E. M. of four to six independent experiments. Stars indicate significant differences from resting level of $[Ca^{2+}]i$ (*$P < .05$; **$P < .01$, student's *t* test).

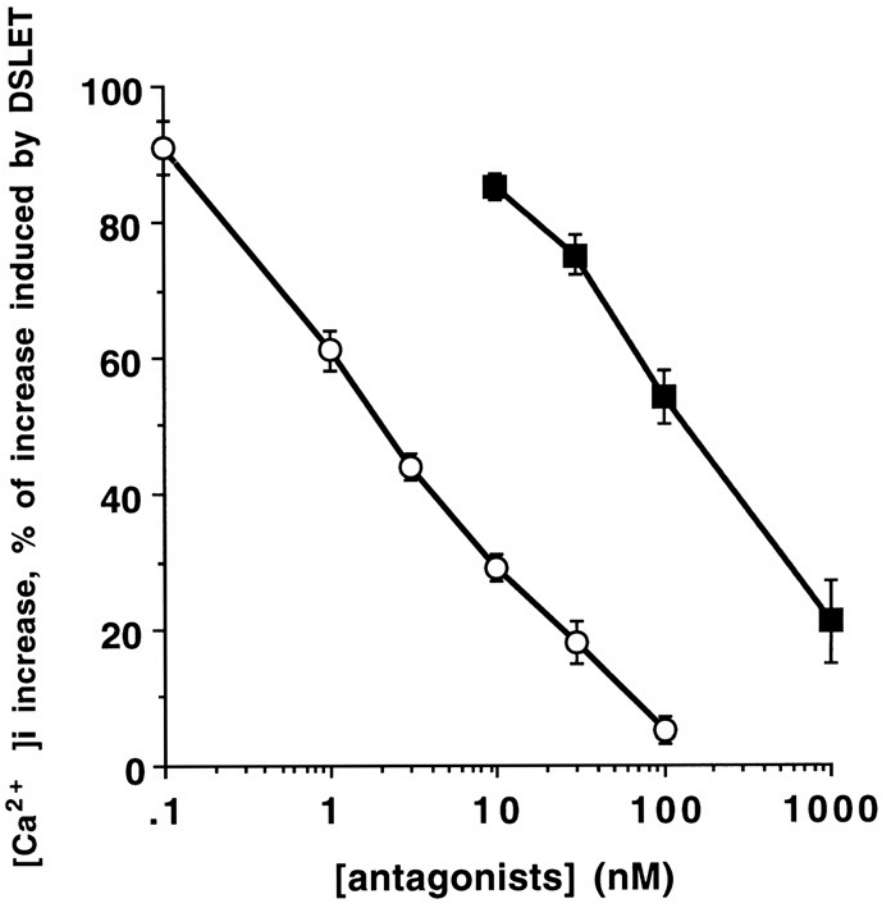


Fig. 5. Inhibitory effects of opioid antagonists, Naltrindole (O) and naloxone (■), on DSLET (100 nM)-induced increase in $[Ca^{2+}]i$ in ND8-47 cells. Values are means ± S. E. M. of three independent experiments at each concentration of the antagonists from 0.1 nM to 1 μM.

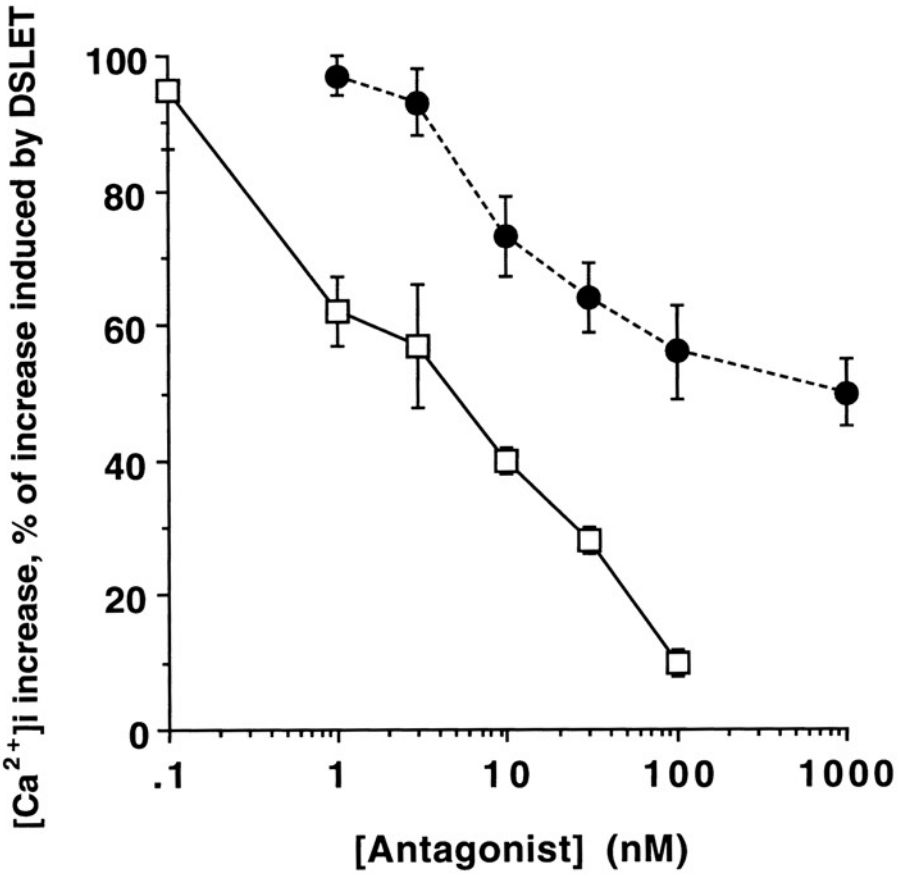


Fig. 6. Inhibitory effects of selective δ antagonists on DSLET-induced increase in $[Ca^{2+}]i$ in ND8-47 cells.
Cells were treated with BNTX (●) and NTB (□), and then were exposed to DSLET (100 nM). Values are means ± S. E. M. of three independent experiments at each concentration of the antagonists from 0.1 nM to 1 μM.

greater potency of deltorphin-II than DPDPE suggest that the increase in $[Ca^{2+}]i$ is induced primarily through the δ_2 subtype of δ-receptor. In analyzing the possible sources of the elevated cytosolic Ca^{2+} after DSLET treatment, ND8-47 cells were transferred into Ca^{2+}-free buffer with 10 mM EGTA to remove any residual extracellular Ca^{2+}. After 60-90 seconds of incubation in Ca^{2+} free buffer, the effects of DSLET (1 nM to 10 μM) were tested. Fig. 7 shows that the removal of external Ca^{2+} reduced the resting $[Ca^{2+}]i$ from 155 nM to115 nM, and completely prevented the DSLET-induced $[Ca^{2+}]i$ increase. These results suggest that the DSLET-induced increase in $[Ca^{2+}]i$ is due to calcium influx. To identify which Ca^{2+} channels were responsible in this process, the voltage-dependent Ca^{2+} channel blockers, nifedipine and verapamil, were used. Fig. 8 show the DSLET-induced increase in $[Ca^{2+}]i$ was inhibited completely by 1 μM nifidipine or 1 μM verapamil and significantly reduced by 1 nM of these agents. This result provides further evidence that the source of the Ca^{2+} is extracellular and indicates that DSLET increases $[Ca^{2+}]i$ by increasing calcium conductance through dihydropyridine-sensitive Ca^{2+} channels.

Fluo-3 study

In order to determine if all cells in the culture responded to opioids with equivalent sensitivity, we used an ACAS 570 laser cytometer to record relative changes in individual cells in the fluorescence of the Ca^{2+}-sensitive intracellular dye, Fluo-3, which is activated at higher excitation wavelength than Fura-2, making it visible in the laser cytometer (Rijkers et al., 1990). The relative

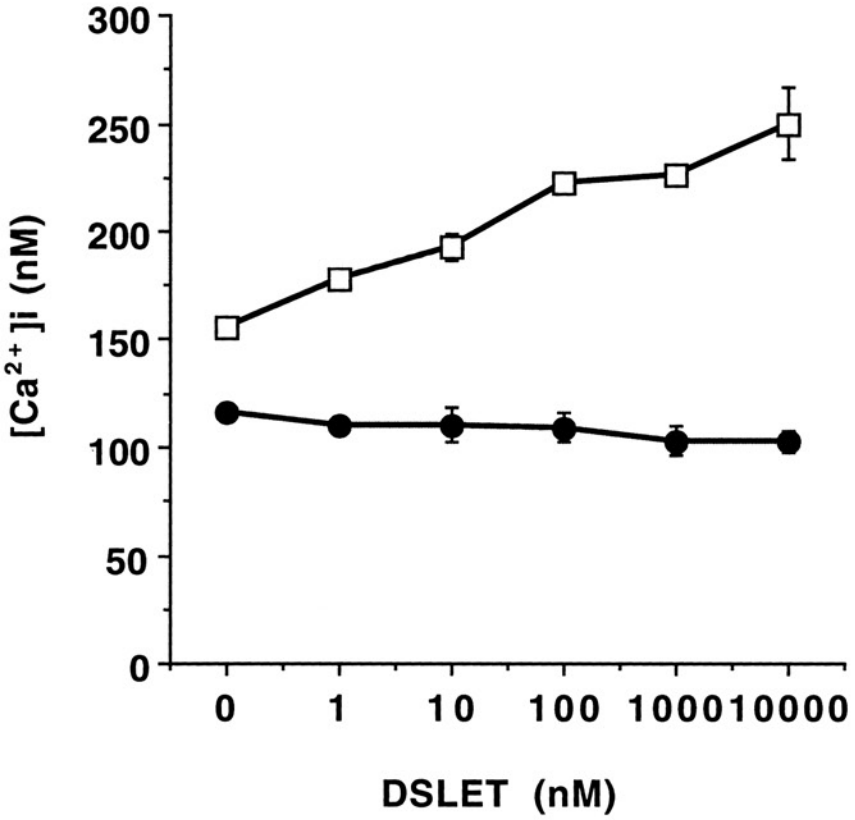


Fig. 7. Effect of removal of external calcium on the DSLET (1 nM to 10 μM)-induced increase in $[Ca^{2+}]i$ in ND8-47 cells.
$[Ca^{2+}]i$ was measured in calcium-containing Na^+ Hanks' solution (□) or calcium-free Na^+ Hanks' solution with 10 mM EGTA (●). The values are means ± S. E. M. of three independent experiments at each concentration of DSLET.

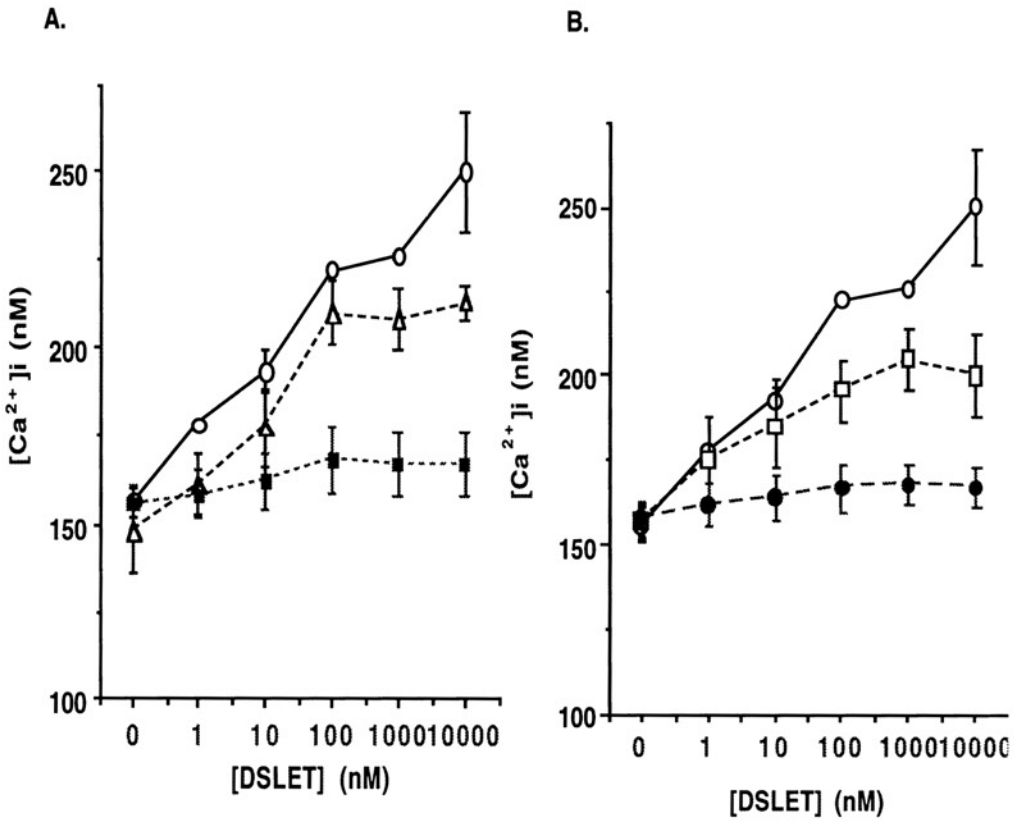


Fig. 8. Effects of calcium channel blockers on DSLET-induced increase in $[Ca^{2+}]i$ in ND8-47 cells.
Cells were treated with the L-type calcium channel blockers, nifedipine (A) and verapamil (B), and then were exposed to DSLET (1 nM to 10 μM). $[Ca^{2+}]i$ was measured in the absence (O), or presence of nifedipine 1 nM (△), 1 μM (■), or verapamil 1 nM (□), 1 μM (●). The values are means ± S. E. M. of three independent experiments at each concentration of DSLET in the absence or presence of calcium channel blockers.

$[Ca^{2+}]i$ change in several ND8-47 cells in each culture was recorded simultaneously. An example of such a scan is shown in Fig. 9. Considering the existence of background noise, a significant $[Ca^{2+}]i$ change was defined as an increase of more than 100 units of the fluorescence intensity (corresponding to 20 nM $[Ca^{2+}]i$) after applications of opioid. There were six cells in the scanning field; five cells responded to 1 μM DSLET with an increase in $[Ca^{2+}]i$. Two of the six cells showed higher resting $[Ca^{2+}]i$; one of those showed a response to 1 μM DSLET. From this type of recording, the fraction of cells responding to each concentration of δ-receptor agonists was calculated (Fig. 10). The fraction of cells responding to agonists was increased when the concentration of δ-receptor agonists was elevated from 100 nM to 10 μM. For DSLET and deltorphin-II, a maximum fractional response was achieved at 1 μM. The percentage of cells responding to DSLET (1 μM), deltorphin-II (1 μM) and DPDPE (1 μM) were 86%, 84%, and 37% respectively. These differences in the fraction of cells responding to each opioid are consistent with the potency order observed in increasing $[Ca^{2+}]i$, as measured by Fura-2. In addition to increasing the fraction of cells showing elevated $[Ca^{2+}]i$, higher concentrations of DSLET, deltorphin-II or DPDPE also resulted in a greater increase in Fluo-3 fluorescent intensity than lower concentrations of the same agents. In cells responding to DPDPE, the increase in Fluo-3 fluorescence was similar for DPDPE, DSLET or deltorphin-II (Fig. 11).

We have also observed the temporal patterns of changes in $[Ca^{2+}]i$ induced by δ-receptor agonists. Upon adding opioids, the $[Ca^{2+}]i$ increased rapidly to a maximum, then gradually returned

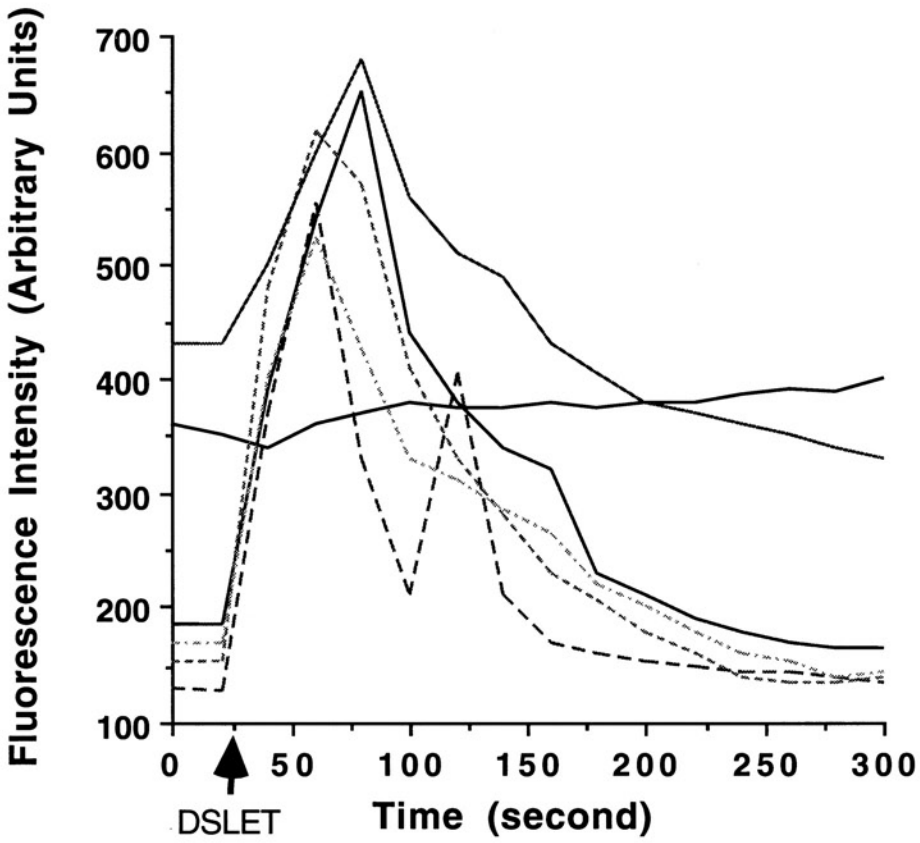


Fig. 9. DSLET-induced increase in single ND8-47 cell.
Six of the cells were recorded simultaneously in one microscope field with an ACAS-570 Argon laser cytometer. DSLET (1 μM) was added at about 20 seconds from the beginning of recording (at arrow). The relative $[Ca^{2+}]i$ changes were reflected as the intensity (in arbitrary units) of Fluo-3 fluorescence in single cells before and during exposure to DSLET.

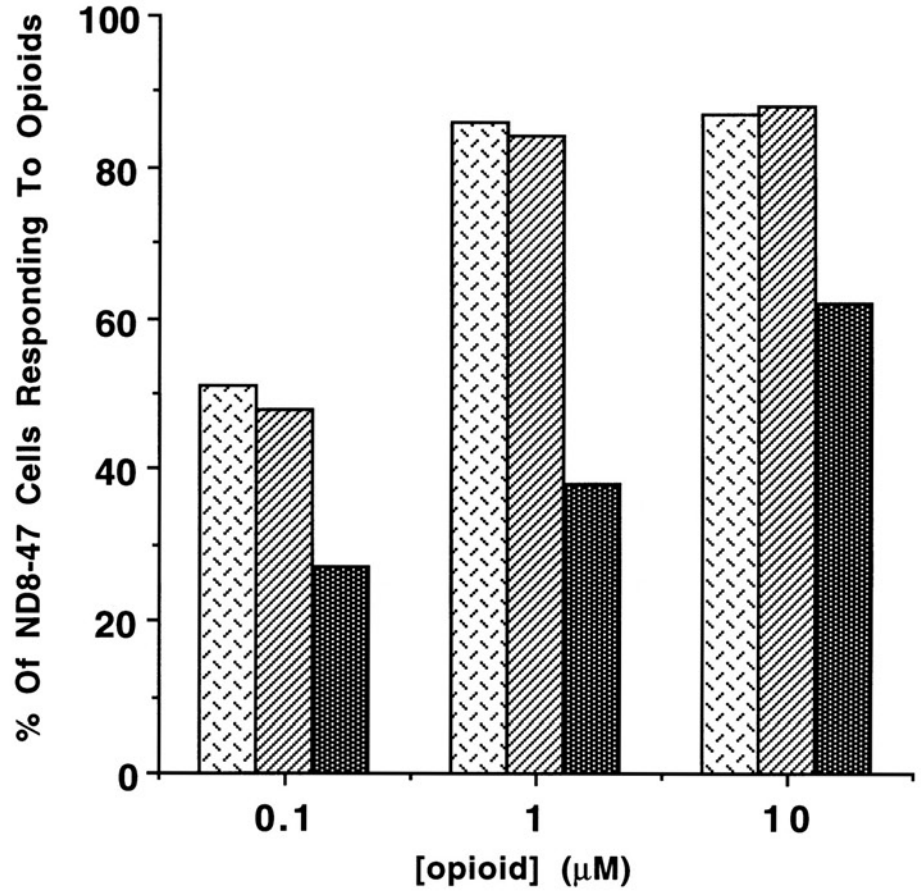


Fig.10 Incidence of ND8-47 cells responding to opioid agonists, DSLET (▨), deltorphin-II (▨) or DPDPE (■).
The percentage of cells showing an increase of 100 or more Fluo-3 fluorescence intensity units after exposure to opioid is recorded. The total numbers of cells recorded at each concentration of opioid were between 30 and 50. The Chi Square test ($P \leq 0.05$) suggests that the fraction of cells responding to agonists was increased when the concentration of δ-receptor agonists was elevated from 100 nM to 10 μM.

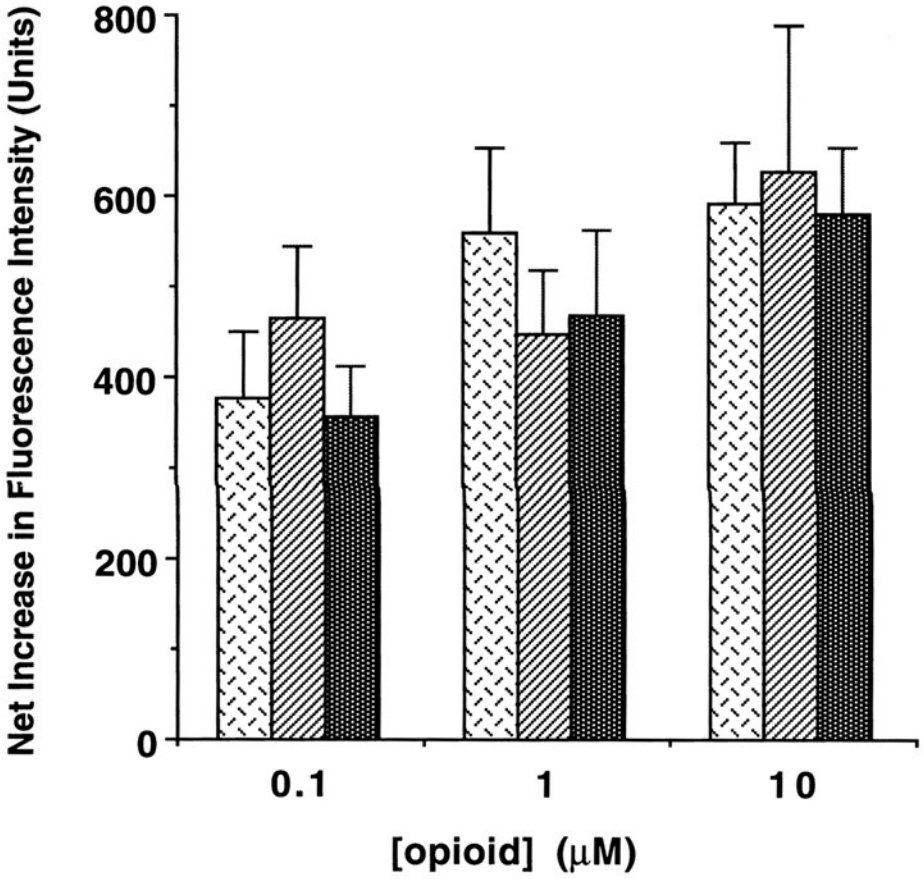


Fig. 11 Net increase in Fluorescence Intensity in ND8-47 cells responding to DSLET (⊠), deltorphin-II(▨), or DPDPE (■). The net increase in Fluorescence Intensity is calculated by subtraction of the resting level of $[Ca^{2+}]i$ from the peak level of $[Ca^{2+}]i$ in those individual cells responding to each opioid. The values are means ± S. E. M. from measurement in 10 to 35 cells responding to each concentration of opioid. The unpaired t test ($P \leq 0.05$) suggests that the fluorescence intensity was significantly increased when the concentration of DSLET or DPDPE was elevated from 100 nM to10 μM.

toward the basal level. The time to peak of $[Ca^{2+}]i$ for DSLET (1 μM), deltorphin-II (1 μM), and DPDPE (1 μM), were 35 ± 2.4 sec (n = 13), 33 ± 2.3 sec (n = 8), and 35 ± 1.7 sec (n = 20), respectively. Different concentrations of opioid agonists had no significant influence on time course of the $[Ca^{2+}]i$ increase.

3. The role of Na^+ channel in opioid induced increase in $[Ca^{2+}]i$

In NG108-15 cells, opioids have been reported to increase $[Ca^{2+}]i$ at least in part by Na^+-influx induced by depolarization of the cell membrane (Jin et al, 1993). Considering this possibility, we tested the effects of the Na^+ channel blocker, tetrodotoxin, and of a reduction in the concentration of extracellular Na^+, on the DSLET response in ND8-47 cells. The results show that those treatments had no effects on DSLET (100 nM)-induced increase in $[Ca^{2+}]i$ (Table 2). Therefore, the opioid-induced Ca^{2+} influx is not likely to result from membrane depolarization following opening of Na^+ channels.

4. The role of intracellular calcium store and the protein kinases in opioid induced increase in $[Ca^{2+}]i$

Although it has been shown that Ca^{2+} influx through dihydropyridine-sensitive Ca^{2+} channel contributes to the opioid-induced increase in $[Ca^{2+}]i$ in ND8-47 cells, the mobilization of intracellular Ca^{2+} may also in part participate in this response. To rule out this possibility, TMB-8, a blocker of Ca^{2+} mobilization from intracellular Ca^{2+} pools (Palade, 1989; Kiang, 1992) was applied to the cells. Pretreatment of cells with TMB-8 (100 μM) for 10 min did not affect the DSLET-induced increase in $[Ca^{2+}]i$ (Fig. 12). This result suggest that DSLET does not cause the release of the intracellular

TABLE 2

Treatment	Resting $[Ca^{2+}]_i$ (nM)	$[Ca^{2+}]_i$ (nM) at DSLET 100 nM
Control	156 ± 7	217 ± 8
Na^+ (0.5 mM)	157 ± 6	208 ± 8
TTX (1 μM)	159 ± 4	211 ± 10

Effect of tetrodotoxin (TTX) and low Na^+ solution on DSLET induced increase in $[Ca^{2+}]_i$ in ND8-47 cells.

Na^+ (0.5 mM) Hank's solution was made by replacement of NaCl with methyl-D-glucamine in Na^+ (145 mM) Hanks' solution. After loading the cells with Fura-2-AM in 145 mM Na^+ Hank's solution, the coverslips were rinsed twice, then placed in the cuvette with 2 ml of low Na^+ (0.5 mM) Hank's solution. Effects of DSLET were tested within 5 min. TTX (1 μM) was added to cells in the cuvette in 145 mM Na^+ Hank's solution 5 min before DSLET was added. Values are means ± S.E.M. of three independent experiments with duplicates in each experiment.

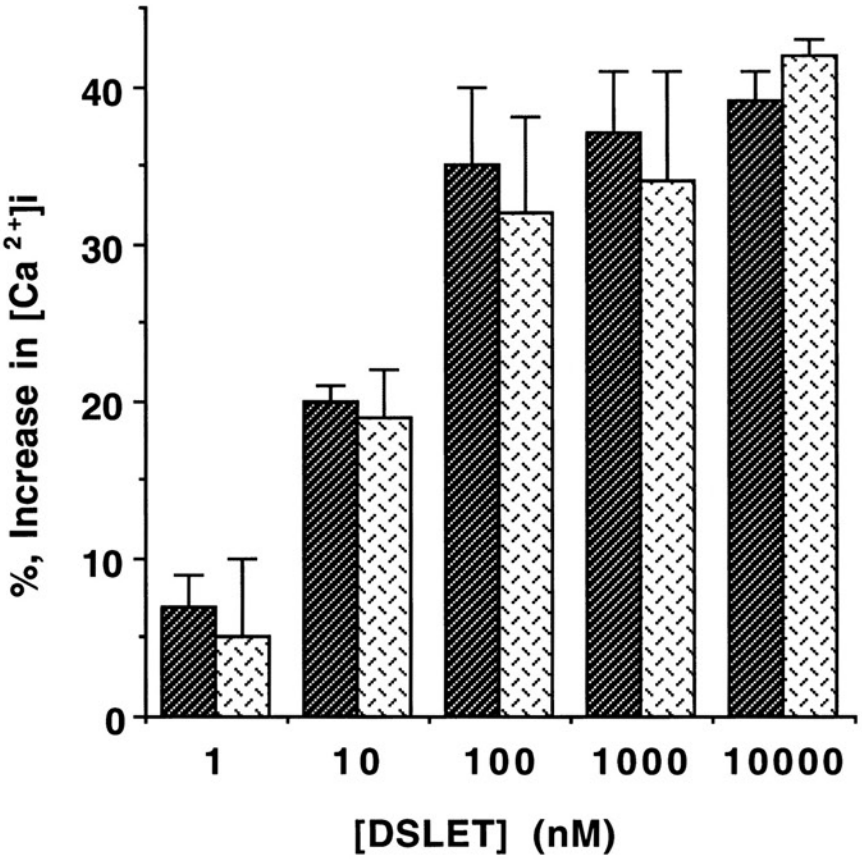


Fig. 12. Effect of TMB-8 on DSLET induced increase in $[Ca^{2+}]i$ in ND8-47 cells.
Cells were untreated (▨) or pretreated with 100 μM TMB-8 for 10 min (☑) before adding DSLET. The values are means ± S. E. M. of three independent experiments at each concentration of DSLET.

calcium from the TMB-8-sensitive stores.

Another potential cause responsible for the increase in $[Ca^{2+}]i$ is that the activation of opioid receptors may stimulate cAMP-dependent protein kinase (PKA) or protein kinase C (PKC), and these enzymes may further phosphorylate calcium channels in the cell membrane or IP_3 receptors (a calcium channel) in the membrane of the intracellular calcium stores. Based on this consideration, I treated the cells with a PKA inhibitor, KT5720 (1 μM) and a PKC inhibitor, calphostin C (1 μM), and examined their effects on DSLET induced-changes in $[Ca^{2+}]i$. As indicated in Fig. 13, KT 5720 and calphostin C did not influence DSLET (100 nM)-induced increase in $[Ca^{2+}]i$. These results suggest that PKA or PKC are not critical factors in the opioid-induced increase in $[Ca^{2+}]i$. No postive controls for the effect of KT 5720 or calphostin C have been conducted in ND8-47 cells; however, at 1 μM, these componds have been reported to inhibit the activity of PKA (Haverstick and Gray, 1992) or PKC in other tissues (Kobayashi et al, 1989), respectively .

5. Effect of opioid on other agent (K^+, Bay K-8644, and bradykinin)-induced increase in $[Ca^{2+}]i$

$\underline{K}^+$: In excitable cells, elevated extracellular concentration of K^+ ($[K^+]_o$) induces membrane depolarization and enhances Ca^{2+} influx through voltage-dependent Ca^{2+} channels. Calcium channels may show different responsiveness to opioids when cell membrane potential is in resting or in depolarized state. We sought to determine if, when the ND8-47 cell membrane is depolarized, opioid

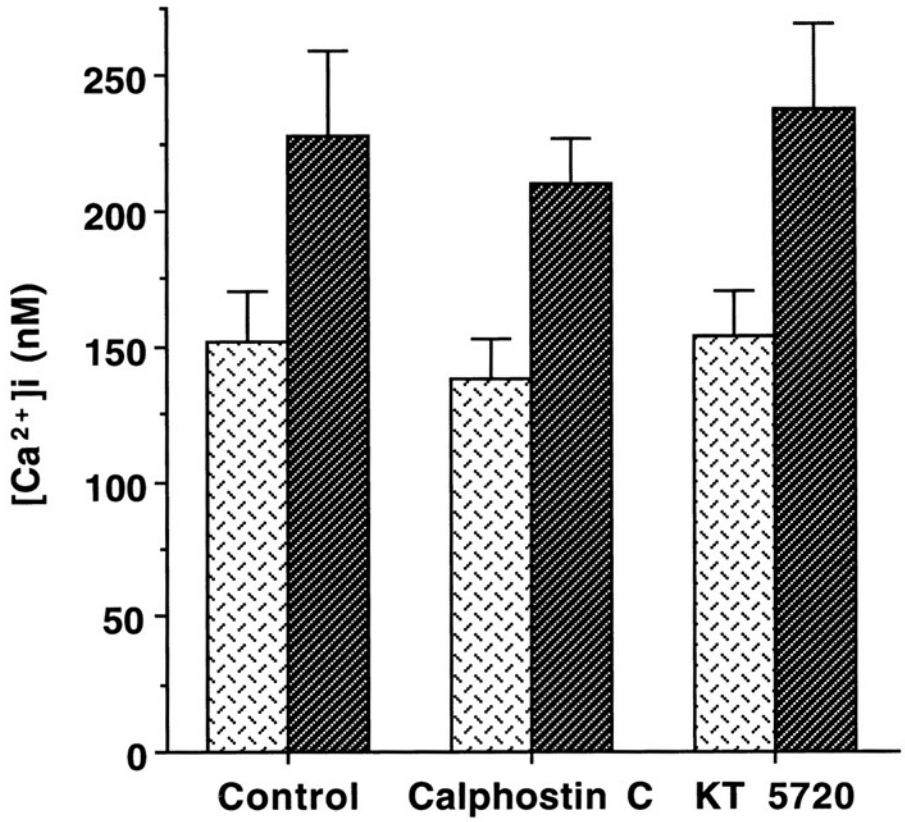


Fig. 13. Effect of KT 5720 and calphostin C on DSLET induced increase in $[Ca^{2+}]i$ in ND8-47 cells.
Cells were pretreated with 1 μM KT 5720 or calphostin C for 10 min. Either of these agents affect the basal $[Ca^{2+}]i$ (▨). Cells were then exposed to 100 nM DSLET (▨). The values are means ± S. E. M. of three independent experiments.

agonist increased Ca^{2+} influx through calcium channels thus elevating $[Ca^{2+}]i$, or whether opioids modify the Ca^{2+} influx induced by membrane depolarization? To address this question, elevated $[K^+]_o$ was applied to ND8-47 cells in the presence or absence of the δ agonist, DSLET. ND8-47 cells were exposed to 1 μM DSLET for 60 to 90 sec followed by adding KCl (15, 25, 40, and 60 mM). As indicated in Fig. 14, KCl induced concentration-dependent increases in $[Ca^{2+}]i$, which were not influenced by treatment of cells with DSLET. This result suggests that the δ agonist, DSLET does not enhance nor inhibit the increase in $[Ca^{2+}]i$ induced by K^+; it only increases the resting level of intracellular calcium.

Bay K-8644: Since the δ agonists induce an increase in $[Ca^{2+}]i$ through the dihydropyridine-sensitive calcium channels in ND8-47 cells, it is important to know the interaction between the opioid binding sites and the dihydropyridine-sensitive calcium channels. The 1, 4-Dihydropyridine, Bay K-8644, is an L-type calcium channel agonist. Its activation of Ca^{2+} current has been reported to be blocked by pertussis toxin and potentiated by GTPγS in cultured rat DRG cells (Dolphin and Scott 1987,1988), which suggest that the effect of Bay K-8644 is probably mediated by a G-protein (G_i or G_o). In ND8-47 cells, Bay K-8644 induced a marked concentration-dependent increase in $[Ca^{2+}]i$, which was not affected by pretreatment of cells with DSLET for either 5 min, or for 24 h (Fig. 15). Thus, the effect of Bay K-8644 and DSLET are not additive during acute exposures. Exposure to DSLET for 24 h (which desensitizes ND8-47 cells to subseqent DSLET exposure; see section

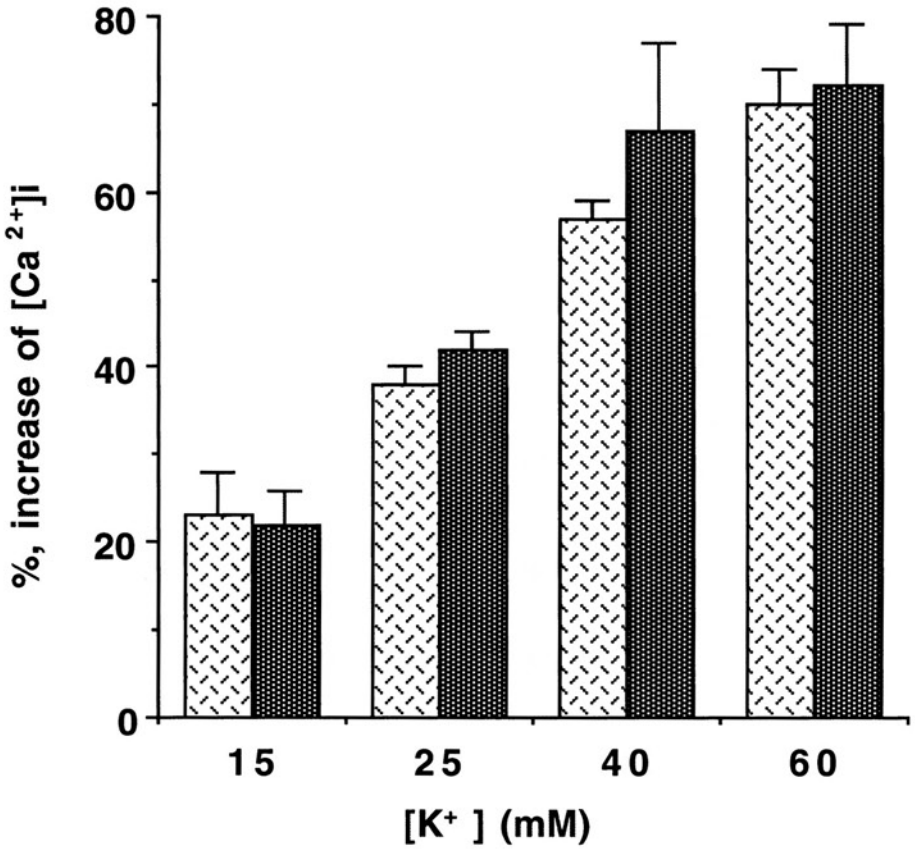


Fig. 14. Effects of DSLET-pretreatment on K^+-induced increase in $[Ca^{2+}]i$ in ND8-47 cells.
Cells were exposed to 1 μM DSLET for 60 to 90 sec followed by concentrated KCl solution (final concentration: 15, 25, 40, and 60 mM) (■). In the control group, cells were exposed to KCl without prior treatment with DSLET (▨). The values are means ± S. E. M. of three independent experiments at each concentration of KCl.

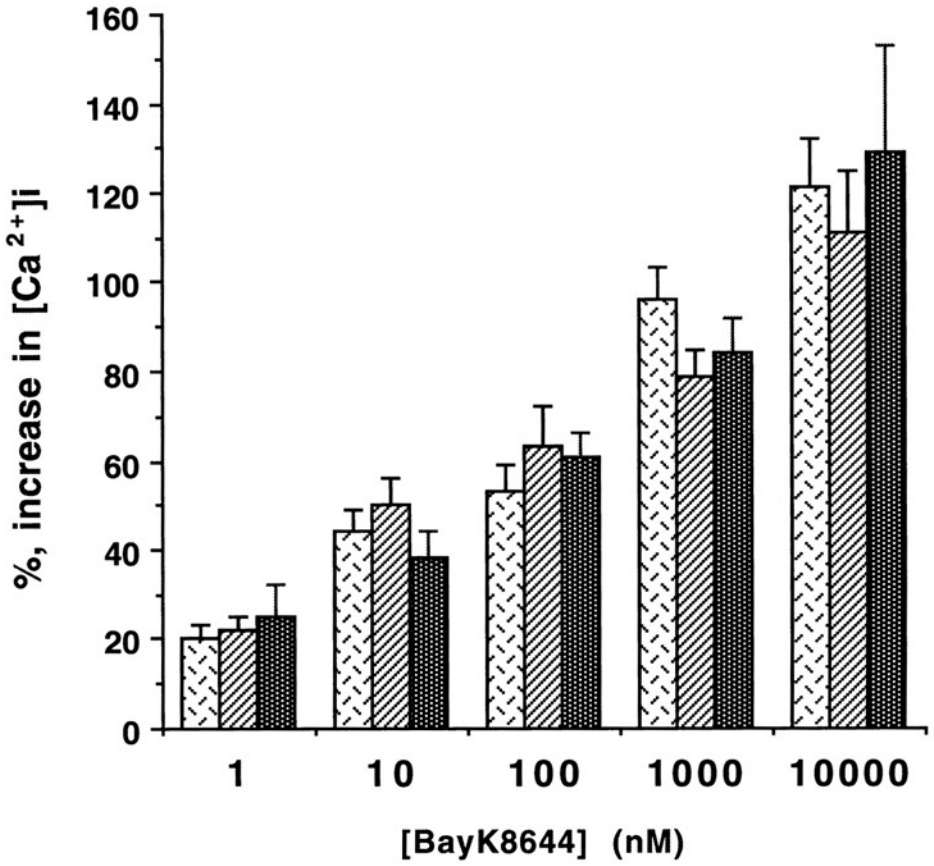


Fig. 15. Effects of DSLET-pretreatment on Bay K-8644-induced increase in $[Ca^{2+}]i$ in ND8-47 cells.
Cells were pretreated with 1 μM DSLET for 5 min (▨) or 24 h (■), then exposed to Bay K-8644 (1 nM - 10 μM). In control group, cells were exposed to Bay K-8644 without prior treatment with DSLET (▨). The values are means ± S. E. M. of three independent experiments at each concentration of Bay K-8644.

III.1) did not inhibit the response to Bay K-8644. The absence of additivity of effect suggests that DSLET and Bay K-8644 may both utilize a common response mechanism. The absence of heterologous desensitization suggests that the initial processes activated by these two agents are different.

Bradykinin: Pretreatment of ND8-47 cells with TMB-8 did not influence DSLET-induced increase in $[Ca^{2+}]i$, indicating that the mobilization of intracellular calcium from TMB-8 sensitive calcium store is not likely to be responsible for the increase in $[Ca^{2+}]i$ induced by opioid. However, evaluating opioid effect on other receptor-mediated mobilization of intracellular calcium is also necessary. It has been reported that activation of bradykinin receptor induces release of Ca^{2+} from internal stores through PLC and IP_3 pathway (Chuang, 1989; Dunn et al., 1991). In addition, it has been known that in other ND cell lines, ND7/23 and ND8, bradykinin can evoke depolarization (Wood et al., 1989; Dunn et al., 1991). As indicated in Fig. 16, bradykinin induced concentration-dependent increases in $[Ca^{2+}]i$, which were not influenced by treatment of cells with DSLET (1 μM). This result suggested that the increases in $[Ca^{2+}]i$ induced by activation of opioid- and bradykinin-receptors are independent pathways; the mobilization of intracellular calcium through the PLC-IP_3 pathway is not likely contribute to the increase in $[Ca^{2+}]i$ induced by δ-opioids.

6. Summary

ND8-47 cells express a high density of opioid δ type receptors. In these cells, δ agonists induce a transient increase in $[Ca^{2+}]i$ with

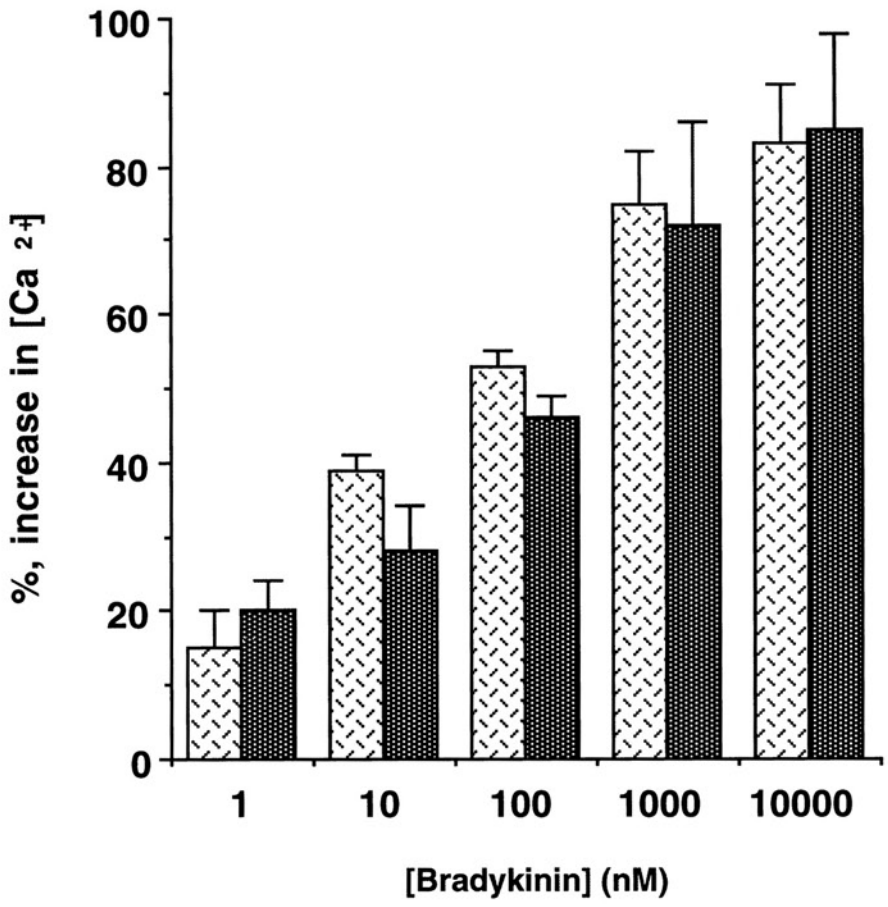


Fig. 16. Effects of DSLET-pretreatment on bradykinin-induced increase in $[Ca^{2+}]i$ in ND8-47 cells.
Cells were pretreated with 1 μM DSLET for 5 min, then were exposed to bradykinin (1 nM - 10 μM) (■). In control group, cells were exposed to bradykinin without prior treatment with DSLET (▨). The values are means ± S. E. M. of three independent experiments at each concentration of bradykinin.

a concentration-dependent fashion; selective μ- and κ-receptor agonists had no effect. This effect was blocked by opioid receptor antagonists, naloxone and naltrindole. Again, the sub-type specific δ-receptor antagonists, 7-benzylidene naltrexone (BNTX; δ_1), and naltriben (NTB; δ_2), were used to characterize further the subtype of δ-receptors mediated this response. NTB was more potent than BNTX in antagonizing the DSLET-induced increase in $[Ca^{2+}]i$, suggesting that the δ_2 receptors are mainly involved in this response. The increase in $[Ca^{2+}]i$ induced by DSLET was blocked by nifedipine (1 μM) or verapamil (1 μM), and was not observed in the absence of external calcium, indicating an opening of dihydropyridine-sensitive L-type calcium channels by the δ agonist. Treatment of cells with DSLET did not influence the increase in $[Ca^{2+}]i$ induced by K^+, Bay K-8644, or bradykinin.

Section II

Identification of G Proteins in Opioid-induced Increase in $[Ca^{2+}]i$

Many studies indicate that the type of G protein coupled with receptors most frequently decides the characters for that receptor mediated response. For example with the opioid receptors, it has been demonstrated that $G\alpha_{i2}$ is mainly responsible for opioid-induced inhibition of adenylyl cyclase activity; G_o is suggested to mediate opioid-induced inhibition of Ca^{2+} channels. Although pertussis toxin-sensitive G proteins have been proposed to mediate opioid-induced mobilization of Ca^{2+} from intracellular stores (Jin et al., 1994) and cholera toxin-sensitive G proteins are suggested to enhance Ca^{2+} channel conductance (Shen and Crain, 1991), the

specific G protein α-subunits implicated in these actions are still unknown. In this study, we used toxins that affect G protein function and an antisense oligonucleotide treatment approach. The results of studies with pertussis toxin and an antisense oligonucleotide to the mRNA for $G\alpha_{i2}$ have shown inhibition of opioid effect on $[Ca^{2+}]i$, suggesting that $G\alpha_{i2}$ is required in opioid-induced increase in $[Ca^{2+}]i$ in ND8-47 cells.

1. Analysis of G protein α subunits in ND8-47 cell membranes

Since the heterogenous G protein subtypes are only different in their α subunits, we examined the presence of G protein α subunits in ND8-47 cell membranes by using Western Blot. G protein α subunits, α_q (41 kDa), α_{i2} (40 kDa), α_{i3} (41 kDa), and α_s (42 kDa and 45 kDa), were detected; α_{i1} (41 kDa) and α_o (39 kDa) were not found in ND8-47 cell membranes (Fig 17 A). In a control experiment, the GC antiserum clearly identified a ~39 kDa band (believed to be α_o) in NG108-15 membranes (conducted by Dr. Thomas Cote) (Fig. 17 B).

2. Effect of pertussis toxin (PTX) and cholera toxin (CT) on opioid induced increase in $[Ca^{2+}]i$

In studies with cultured DRG neurons, treatment of DRG cells with PTX (1 μg/ml) or CT (10 nM) for 1 day can block opioid-induced (at μM concentration) shortening of the action potential duration (APD) or opioid-induced (nM concentration) prolonging of APD, respectively (Shen and Crain, 1989). In my study with ND8-47 cells, incubation of cells with PTX at 0.1 μg/ml for 24 hours almost completely blocked the DSLET-induced increase in $[Ca^{2+}]i$ (Fig. 18). Incubation of cells with cholera toxin at 10 nM for 24 hours had no

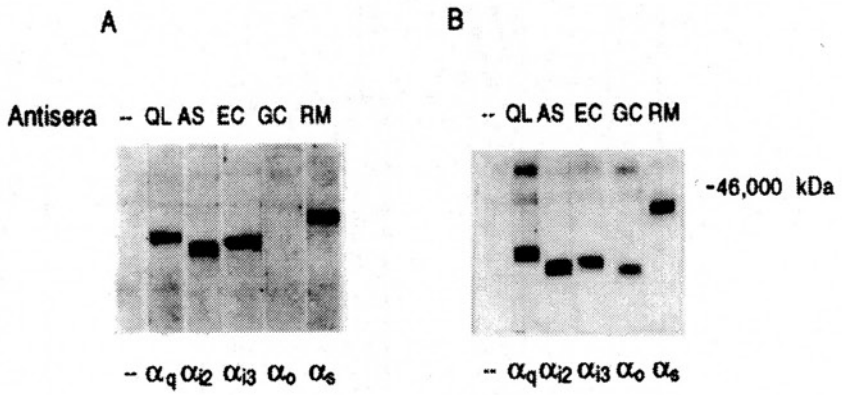


Fig. 17. Western blot of G protein α subunits in ND8-47 cell membranes (A) and NG108-15 cell membranes (B).
G proteins from cell membranes (20 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose and incubated with the following antisera: QL (selective for α_q) at 1:500; AS (selective for α_{i1} and α_{i2}) at 1:4,000; GC (selective for α_o) at 1:1,000; EC (selective for α_{i3}) at 1:1,000; and RM (selective for α_s) at 1:4,000.

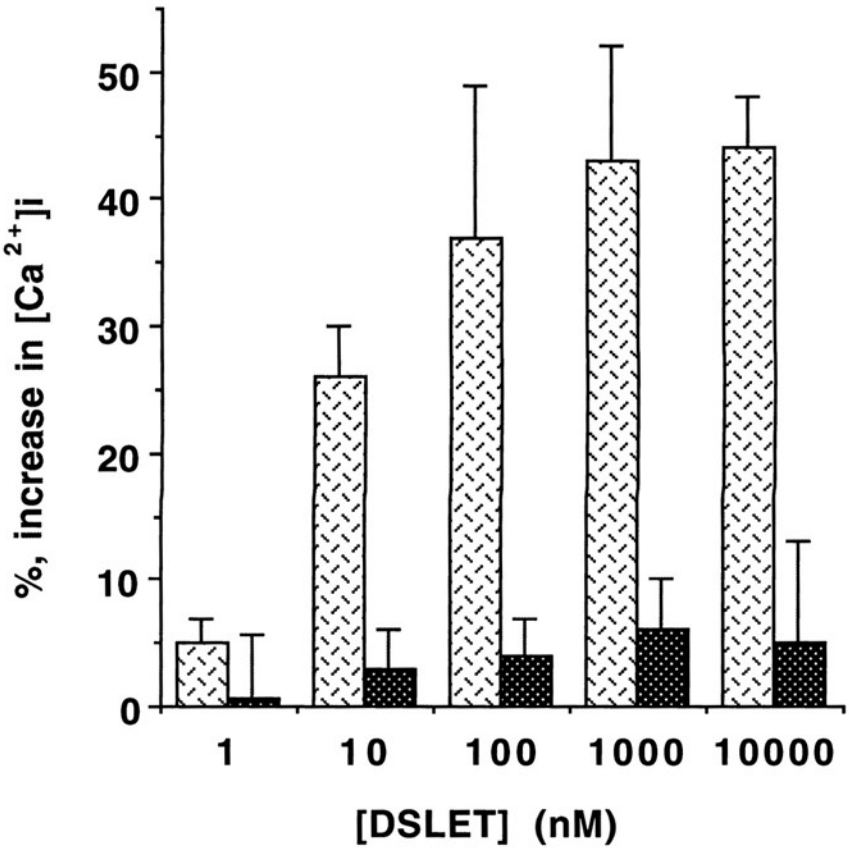


Fig. 18. Effect of pertussis toxin (PTX) on DSLET induced increase in $[Ca^{2+}]i$ in ND8-47 cells.
Cells were incubated with 0.1 μg/ml PTX for 24 hr, and then they were exposed to DSLET (1-10^5 nM) (■). In control group, cells were exposed to DSLET without prior treatment with PTX (⊡). The values are means ± S. E. M. of three independent experiments at each concentration of DSLET.

effect on the DSLET-induced increase in $[Ca^{2+}]i$ (Fig. 19). These results suggest that the PTX sensitive G proteins, G_i or G_o are involved in the opioid induced increase in $[Ca^{2+}]i$ in ND8-47 cells; CT sensitive Gs is probably not implicated.

3. Effect of pertussis toxin (PTX) or GTPγS on opioid receptor binding

Treatment of cells with PTX (0.1 μM) for 24 hours or treatment of cell membranes with GTPγS (10 μM) for 30 min significantly decreased agonist affinity ($1/K_i$) to opioid receptors without changing receptors density (Bmax) (Table 3) when the data were fitted into a one binding site model. Agonist binding to ND8-47 cell membranes was best fit by a two binding site model. Analysis using a two-binding site model, which was significantly better than the one-site model by F-test ($p \leq 0.05$) showed that in the absence of PTX or GTPγS, most agonist binding sites are in a high affinity state ($K_i \approx 1$ nM), while in the presence of PTX or GTPγS most agonist binding sites are in a low affinity state ($K_i \approx 20$ nM). These results suggest uncoupling of G protein to the receptor that is due to the ribosylation of G_i/G_o protein by pertussis toxin or the binding of GTP to the G protein.

4. Effect of antisense oligonucleotide treatment

Generally, living cells are capable of taking up short oligonuceotides (Tortora, et al., 1990, 1991; Yokozaki, et al., 1993). In this study, the oligonucleotides were delivered into the cells by incubation of the cells with 10 μM oligonucleotides for 2, 4 and 6 days. The effects of treatment with antisense oligonucleotides to α_{i2}, α_{i3}, and α_s on δ-agonist-induced increase in $[Ca^{2+}]_i$ in ND8-47

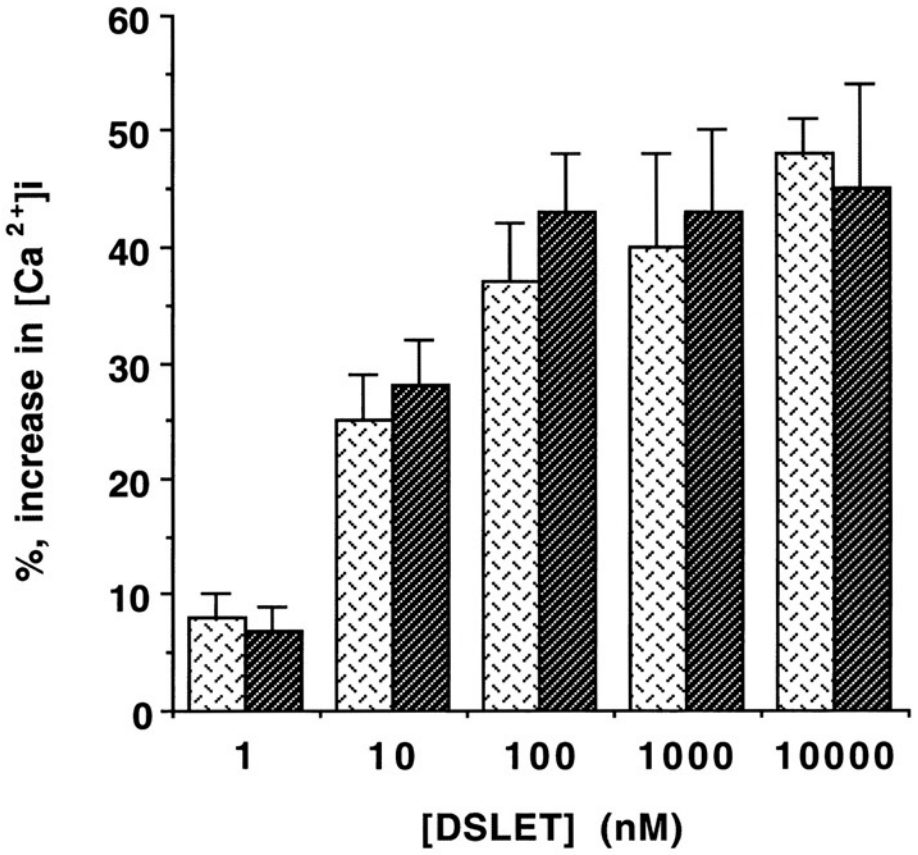


Fig. 19. Effects of cholera toxin (CT) on DSLET induced increase in $[Ca^{2+}]i$ in ND8-47 cells.
Cells were incubated with 10 nM CT for 24 hr, and then were exposed to DSLET (1-10^5 nM) (■). In control group, cells were exposed to DSLET without prior treatment with PTX (▨). The values are means ± S. E. M. of three independent experiments at each concentration of DSLET.

TABLE 3

GTPγS or PTX	No. of independent assays	Assumed No. of binding site	Ki (%CV) nM	B_{max}(%CV) fmol/mg protein	% of sites in high affinity form	Curve fitting F-test
Control	6	1	5.7 (22%)	330 (8%)	-	-
		2	1.3 (110%) (K_iH) 22 (97%) (K_iL)	320 (45%) 59 (119%)	84	P=0.006
GTPγS teatment	3	1	13 (36%)	330 (13%)	-	-
		2	0.72 (250%) (K_iH) 18 (93%) (K_iL)	34 (180%) 300 (69%)	11	P=0.03
PTX treatment	3	1	19 (23%)	310 (7%)	-	-
		2	0.79 (110%)(K_iH) 21 (210%) (K_iL)	87 (190%) 310 (54%)	22	P=0.05

Competition for [^{3}H]DIP binding by unlabeled DSLET in membranes from untreated, or PTX or GTPγS treated ND8-47 cells.

In PTX treated samples, membranes were made directly from cells that were cultured with 0.1 μg/ml PTX for 24 hr. In GTPγS treated samples, membranes from untreated cells were incubated for 30 min at 37°C in Krebs-HEPES buffer in presence of 10 μM GTPγS before addition of competing ligand and [^{3}H]DIP, as described in Methods. K_iH and K_iL are the dissocation constants of high affinity and low affinity binding sites in a two-site model. The values are means (%CV) of three independent experiments. %CV is a percentage coefficient of variation (standard error divided by the parameter value, See Munson & Rodbard, 1980). The goodness of fit for a two-site model was compaired to a one-site model by F-test. For control and GTPγS treated membranes, the experimental data were fitted significantly better by a two-site model ($P< 0.05$). For the PTX-treated cells, P=0.05. We therefore regard the two-site model as best fitting the data under all conditions. To confirm that the treatment significantly changed the binding parameters best fitting the data, the parameters derived under control conditions (two-site model) were tested for goodness-of-fit to the treated sample data. In each case the control parameter yielded a significantly less satisfactory fit (F-test, $P<0.01$) than the parameters estimated independently for each treatment (as indicated in the Table).

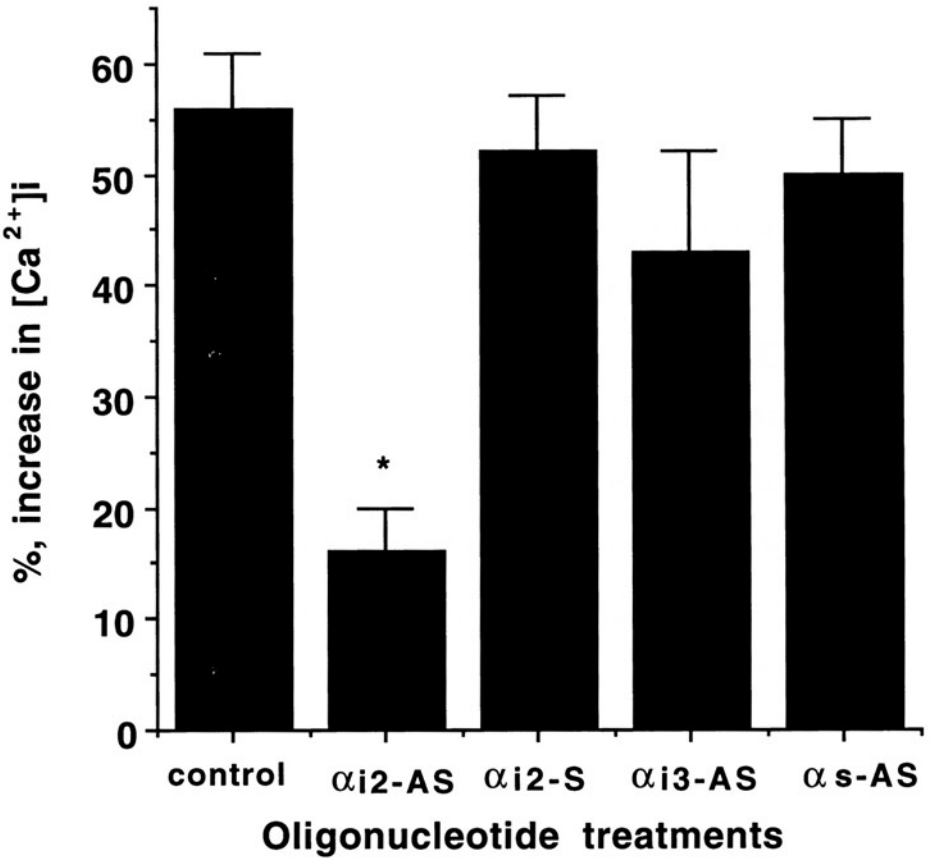


Fig. 20. Effects of Antisense Oligos (against G proteins) on DSLET-induced Increase in $[Ca^{2+}]i$ in ND8-47 cells.
Changes in $[Ca^{2+}]i$ induced by 100 nM DSLET in untreated cells (control) or cells treated with 10 μM antisense (α_{i2}-AS, α_{i3}-AS, α_s-AS) or sense oligonucleotides (α_{i2}-S) for 6 days. Each bar represents the mean ± SEM of three independent experiments using a statistic program SuperANOVA 1.01(* $P < 0.05$). The resting level of $[Ca^{2+}]i$ did not change significantly after exposure of cells to oligonucleotides; the $[Ca^{2+}]i$ was 148 ± 12 nM in untreated cells; 155 ± 8 nM in α_{i2}-AS treated cells; 150 ± 14 nM in α_{i3}-AS treated cells; 168 ± 13 nM in αs-AS treated cells; and 134 ± 16 nM in α_{i2}-S treated cells.

cells are shown in Fig. 20. Incubation of cells with 10 μM α_{i2}-AS (antisense to α_{i2}) for 6 days resulted in 73% inhibition of DSLET (100 nM)-induced increase in $[Ca^{2+}]_i$. In contrast, α_{i3}-AS (antisense to α_{i3}), αs-AS (antisense to αs) and α_{i2}-S (sense to α_{i2}) treatment for the same time had no significant influence on DSLET-induced response. Treatment of cells with α_{i2}-AS for 4 days induced a 25% inhibition of DSLET (100 nM)-induced increase in $[Ca^{2+}]_i$; however, treatment for two days did not influence the DSLET action (Fig. 21).

These results suggest that the inhibitory effect of α_{i2}-AS on the DSLET-induced increase in $[Ca^{2+}]_i$ was due to the inhibition of $G\alpha_{i2}$ expression. To examine the changes in the levels of G proteins after antisense treatment, we treated ND8-47 cells with 10 μM α_{i2}-AS for 6 days. The presence of G protein α subunits ($G\alpha_i$, $G\alpha_o$, $G\alpha_q$, and $G\alpha_s$) was examined by Western blot analysis in membranes from untreated cells (Fig. 22 A) or from antisense-treated cells (Fig. 22 B). G protein α subunits α_q (41 kDa), α_{i2} (40 kDa), α_{i3} (41 kDa), and αs (42 kDa and 45 kDa) were detected; α_{i1} (41 kDa) and α_o (39 kDa) were not detected in either treated or untreated ND8-47 cell membranes. Comparing to untreated cells, there was an apparent decline in the level of α_{i2} after the cells were treated with α_{i2}-AS for 6 days, while the level of α_{i3}, α_q, α_s were not changed. The time-course for the change in the level of α_{i2} was also examined (Fig. 23 A). The level of α_{i2} declined from 4-day treatment with α_{i2}-AS and was reduced more markedly after treatment for 6 days. This time-dependent inhibition of α_{i2} expression by α_{i2}-AS is parallel to the time-dependent inhibition of the DSLET-induced increase in $[Ca^{2+}]_i$ by α_{i2}-AS.

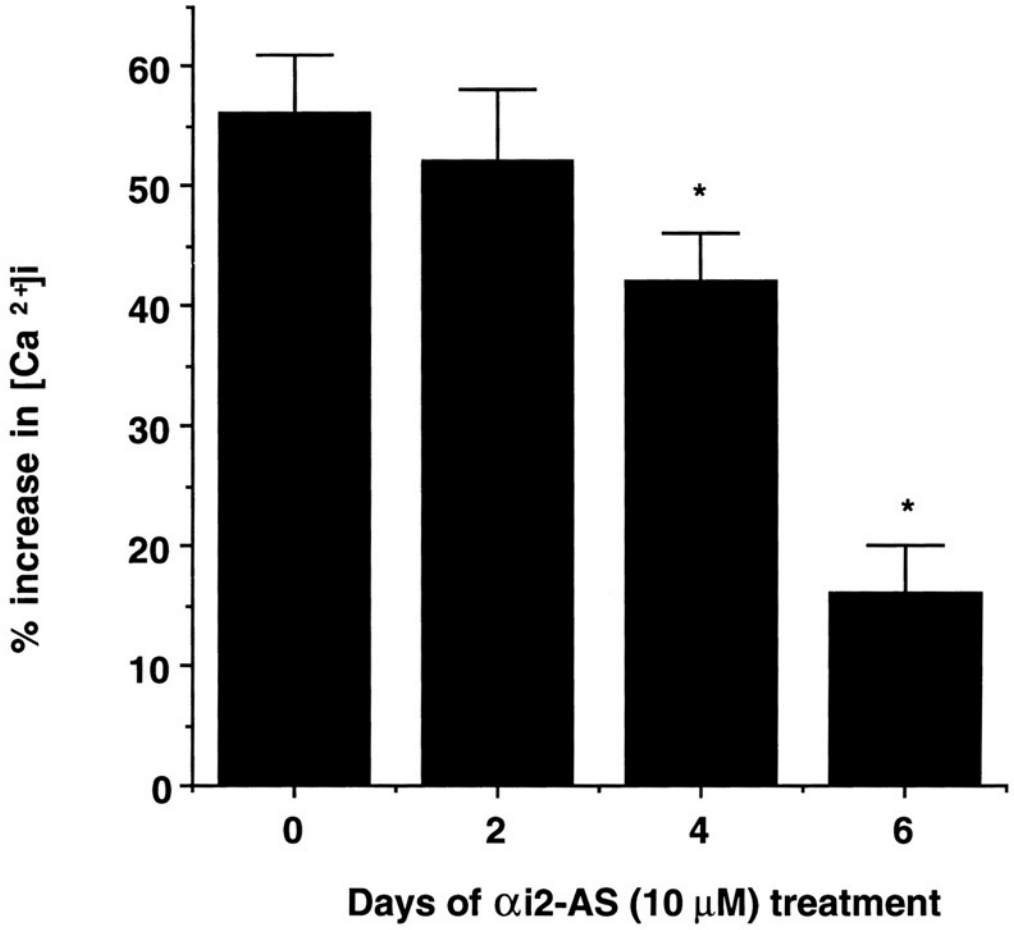


Fig. 21. Effect of Antisense Oligonucleotide (against $G\alpha_{i2}$) on DSLET-induced Increase in $[Ca^{2+}]i$.
DSLET-induced changes in $[Ca^{2+}]i$ were measured in untreated cells and the cells pretreated with 10 μM α_{i2}-AS for 2, 4, and 6 days. Each bar represents the mean ± SEM of three independent experiments using a statistic program SuperANOVA1.01(* $P < 0.05$).

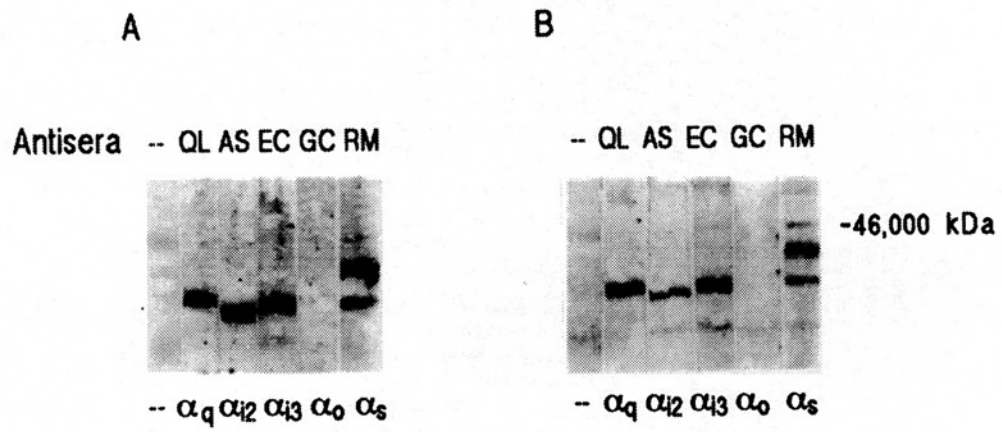


Fig. 22. Western blot of G protein α-subunits in membranes of untreated ND8-47 cells (A) and ND8-47 cells treated with 10 μM α_{i2}-AS for 6 days (B).
G proteins from cell membranes (20 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose and incubated with the following antisera: QL (selective for αq) at 1:500; AS (selective for αi_1 and αi2) at 1:4,000; GC (selective for αo) at 1:1,000; EC (selective for αi3) at 1:1,000; and RM (selective for αs) at 1:4,000. To analyze these results, Western blots were scanned with a laser densitomiter. The areas under each α-subunits image density peak were measured using the program NIH Image v. 1.55. Mean values (square pixels) ± S.E. for each band were calculated from three independent experiments using a statistical program SuperANOVA 1.01 (the figure shows a representitive experiment). After αi2-AS treatment for 6 days, the intensity of the αi2 band was significantly reduced (* $P < 0.05$), while the intensity of the αq, αi3, and αs bands were unchanged.

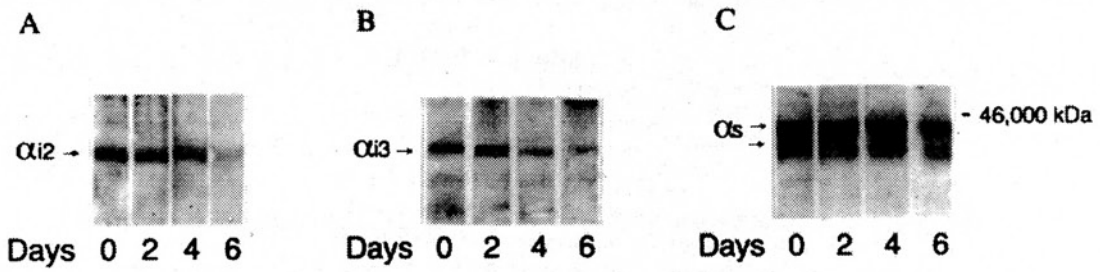


Fig. 23. Western blot of G protein α-subunits in membranes of ND8-47 cells treated with antisense oligonucleotides to α_{i2}, α_{i3}, or α_s. Cells were treated with 10 μM of α_{i2}-AS (A), α_{i3}-AS (B), and α_s-AS (C) for 2, 4, and 6 days. G proteins from cell membranes (20 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose and incubated with the following antisera: AS (selective for α_{i1} and α_{i2}) at 1:4,000 (A); EC (selective for α_{i3}) at 1:1,000 (B); and RM (selective for α_s) at 1:4,000 (C). The results were analyzed by scanning the blots using a laser densitometer. The intensity of the α_{i2} and α_{i3} bands began to decline after antisense-treatment for 4 days; the reduction was even more apparent after treatment for 6 days. The intensity for α_s was reduced almost to a half after treating cells with antisense against α_s for 6 days. Note that α_s appears as a doublet of MW 45 kDa and MW 42 kDa with a high amount of the higher molecular weight form. Antisense oligonucleotide treatment against α_s reduced the amount of both forms.

Since treatments of ND8-47 cells with antisense oligonucleotide against $G\alpha_{i3}$ or $G\alpha_s$ did not influence the DSLET-induced increase in $[Ca^{2+}]_i$, we examined how α_{i3}-AS or α_s-AS affected the expression of either α_{i3} or α_s in the ND8-47 cell membranes (Fig. 23 B, C). After treatment of cells with antisense oligonucleotides for 4 days, the levels of α_{i3}, or α_s began to decline; the reduction was even more apparent after treatment for 6 days. These results indicate that G proteins $G\alpha_{i3}$ or $G\alpha_s$ probably do not participate in opioid regulation of intracellular calcium in ND8-47 cells.

5. Summary

The δ-pioid agonist (DSLET)-induced increase in $[Ca^{2+}]i$ through a pertussis toxin sensitive mechanism. It is clear that ND8-47 cells, in contrast to NG108-15 cells, do not have $G\alpha_o$ subunits which seem to be important for the inhibition of calcium channels by opioid receptors. This fact may account for the absence of inhibition of calcium channels or reduction in $[Ca^{2+}]_i$ by opioid receptors. Using an antisense oligonucleotide treatment approach, we have demonstrated that the increase in intracellular calcium levels induced in ND8-47 cells by activation of δ type opioid receptors is inhibited by prior treatment of the cells with antisense oligonucleotide directed against G protein α_{i2}-subunit, but not by the complementary sense oligonucleotide, or antisense oligonucleotides to other G protein α-subunits present in the membranes. Evidence presented here suggests that opioid receptor activation of dihydropyridine-sensitive calcium channels is mediated by $G\alpha_{i2}$ protein through an as yet undefined mechanism.

Section III
Effects of chronic Opioid Treatment in ND8-47 Cells

Opioid tolerance induced by sontinuous treatment is a complicated process. The studies on the effects of chronic opioid treament of NG108-15 and 7315c cells revealed a process involving an initial loss of agonist-induced inhibition of adenylyl cyclase activity (desensitization) and a later reduction in the number of receptors (down-regulation) (Law et al., 1982, 1983; Puttfarcken et al., 1988, 1989). Chronic opioid treatment reduces the coupling state of the receptor to G protein, and may also alter the G proteins levels in certain tissues (Attali and Vogel, 1989; Long and Costa, 1989). So far, there are no studies reported in any neuronal tissues about the opioid-induced changes in $[Ca^{2+}]i$ during sustained opioid exposure. The following studies focused on the changes of receptor binding and opiate regulation of $[Ca^{2+}]i$ in cells persistently exposed to opioid agonist.

1. Opioid regulation of $[Ca^{2+}]i$ in cells chronically exposed to the δ agonist

Chronic opioid treatment caused marked desensitization of the opioid-induced increase in $[Ca^{2+}]_i$. Pretreatment of the cells with DSLET (1 nM - 10 μM) for 24 hr induced a concentration-dependent desensitization of the acute effect of DSLET on $[Ca^{2+}]_i$ (Fig. 24). Pretreatment with DSLET at 10 nM begins showing a significant desensitization, and 1 μM DSLET pretreatment shows about 80%

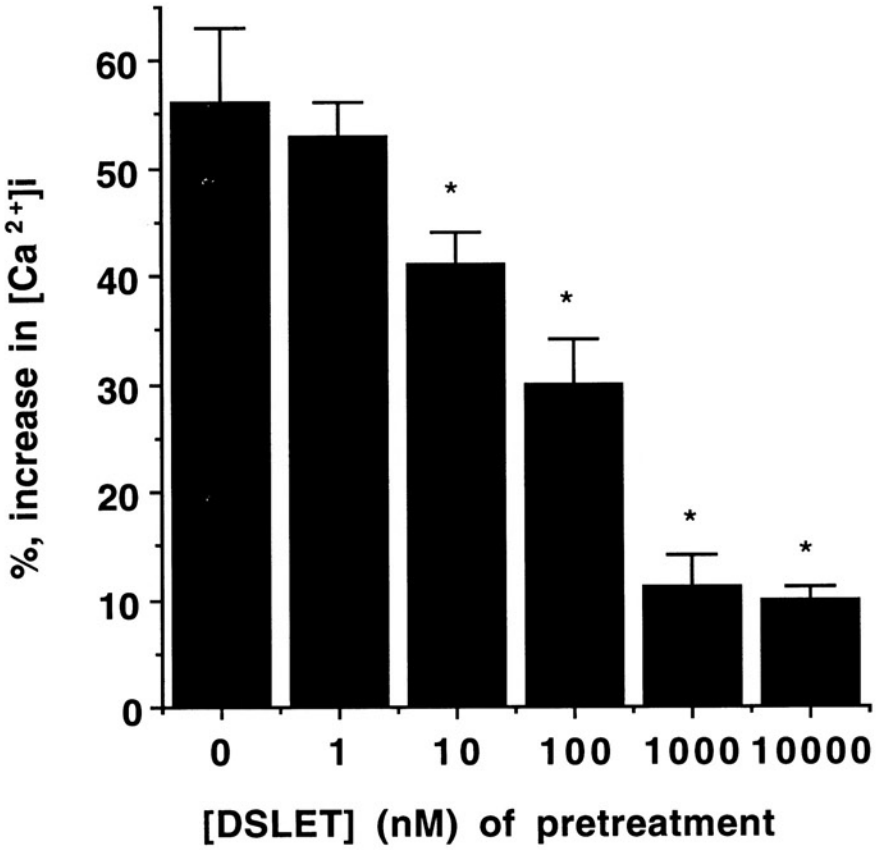


Fig. 24. DSLET effect on $[Ca^{2+}]i$ in ND8-47 cells pretreated with DSLET (1-10^5 nM).
After cultured with DSLET (1-10^5 nM) for 24 hr, cells were washed 5 times with Kreb's buffer, then 100 nM DSLET were added and $[Ca^{2+}]i$ were measured immediately. Values are means ± S.E.M. of three independent experiments (* $P < 0.05$, student *t* test).

inhibition of DSLET induced response. This effect was also time-dependent. Pretreatment of the cells with DSLET (1 μM) from 0.5 to 24 hr progressively reduced the acute effect of DSLET on $[Ca^{2+}]_i$ (Fig. 25). A significant desensitization was observed after 1 hr of DSLET treatment. Maximum desensitization was achieved by 6 or more hours of DSLET-pretreatment, which induced more than 70% inhibition of the DSLET-induced increase in $[Ca^{2+}]_i$. Pretreatment of cells by another δ agonist, DPDPE (1 nM - 10 μM), also induced a significant concentration-dependent desensitization of the acute effect of DSLET on $[Ca^{2+}]_i$; 1 μM DPDPE pretreatment for 24 hr resulted in approximately 60% inhibition of DSLET induced-increase in $[Ca^{2+}]_i$ (Fig. 26).

2. Chronic opioid effect on opioid receptor binding

A reduction of opioid agonist affinity and/or receptor down regulation may be the principle cause(s) for the loss of opioid responses. Table 4 shows the opioid antagonist diprenorphine (DIP) competing with [^{3}H] DIP binding in membranes from cells that were treated with DSLET for 6 and 24 hr. DSLET pretreatment for 6 and 24h reduced receptor density by 24% and 70% respectively without significant change of affinity to DIP. Under chronic opioid treatment, opioid agonist binding assays show both changes of affinity and receptor numbers (Table 5). For control samples and DSLET-treated (1 hr or 6 hr) samples, a two-site model was significantly better than a one-site model by F-test ($p \leq 0.05$). The two-site model did not fit the 24 hr treated samples better than an one-site model, probably as a result of the marked reduction in

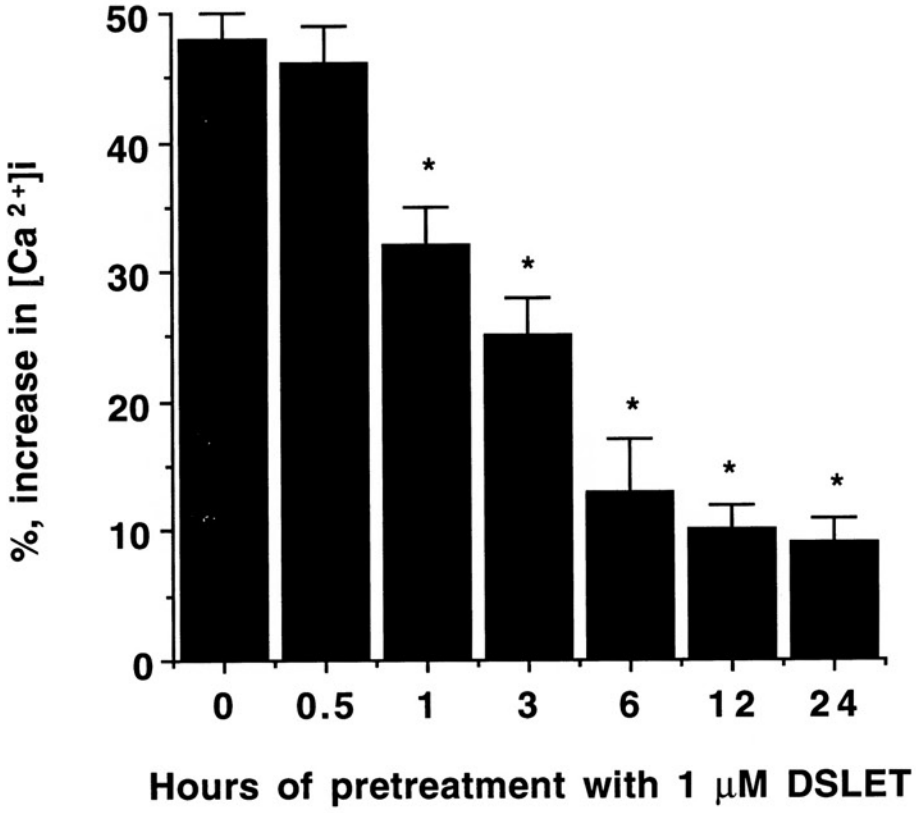


Fig. 25. DSLET effect on $[Ca^{2+}]i$ in ND8-47 cells pretreated with DSLET for 0.5 - 24 h.
After cultured with DSLET 1 μM for 0.5 - 24 hr, cells were washed 5 times with Kreb's buffer, then 100 nM DSLET were added and $[Ca^{2+}]i$ were measured immediately. Values are means ± S.E.M. of three independent experiments (* $P < 0.05$, student *t* test).

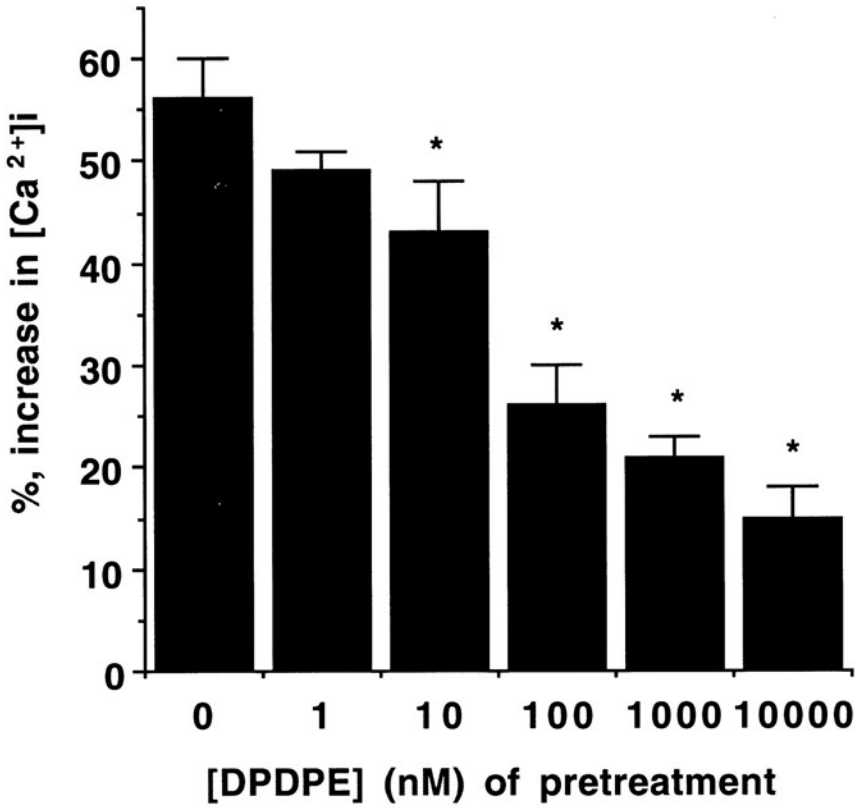


Fig. 26. DSLET effect on $[Ca^{2+}]i$ in ND8-47 cells pretreated with DPDPE (1 - 10^5 nM).
After cultured with DPDPE (1-10^5 nM) for 24 hr, cells were washed 5 times with Kreb's buffer, then 100 nM DSLET were added and $[Ca^{2+}]i$ were measured immediately. Values are means ± S.E.M. of three independent experiments (* $P < 0.05$, student *t* test).

TABLE 4

Hours of DSLET (1μM) pretreatment	No. of independent assays	Ki (%CV) (nM)	B_{max} (%CV) (fmol/mg protein)
Untreated	6	0.78 (77%)	459 (24%)
6	3	0.95 (68%)	337 (49%)
24	3	0.70 (85%)	130 (34%)

Competition for [^{3}H]diprenorphin (DIP) (1 nM) binding by unlabeled diprenorphin in membranes from control and treated ND8-47 cells exposed to DSLET for 6, and 24 hr.
B_{max} and K_i values were estimated from the pooled data by non-linear regression (ligand) in a one-site model. The values are means (%CV) of three independent experiments. %CV is a percentage coefficient of variation (standard error divided by the parameter value, see Munson & Rodbard, 1980).

TABLE 5

Hours of DSLET treatment	No. of independent assays	Assumed No. of binding site	Ki (%CV) nM	B_{max} (%CV) fmol/mg protein	% of sites in high affinity form	Curve fitting F-test
Untreated (control)	10	1	6.6 (14%)	350 (6%)	-	-
		2	2.8 (22%) (K_{iH}) 24 (86%) (K_{iL})	350 (6%) 31 (41%)	92	P=0.004
1	4	1	12 (14%)	330 (11%)	-	-
		2	1.7 (120%) (K_{iH}) 29 (250%) (K_{iL})	170 (78%) 190 (140%)	47	P=0.044
6	4	1	16 (57%)	78 (3%)	-	-
		2	0.82 (270%) (K_{iH}) 19 (21%) (K_{iL})	9 (76%) 73 (22%)	11	P=0.038
24	7	1	18 (14%)	35 (3%)	-	-

Competition for [^{3}H]DIP (1 nM) binding by unlabeled DSLET in membranes from control and from treated ND8-47 cells exposed to DSLET (1 μM) for 1, 6, and 24 hr. K_{iH} and K_{iL} are the dissociation constants of high affinity and low affinity binding sites in a two-site model. The values are means (%CV) of 4 - 10 independent experiments. %CV is a percentage coefficient of variation (standard error divided by the parameter value, see Munson & Rodbard, 1980). The goodness of fit for a two-site model was compaired to a one-site model by F-test. For control and DSLET 1 and 6 hr treated membranes, the experimental data was fitted significantly better by a two-site model ($P < 0.05$) than a one-site model. For the 24 hr treated samples, it was not possible to fit the data to a two-site model because of the substantial reduction in the number of binding sites. To confirm that the treatments significantly changed the binding parameters best fitting the data, the parameters derived under control conditions (two-site model) were tested for goodness-of-fit to the each of the treated sample data sets. In each case the control parameter yielded a significantly less satisfactory fit (F-test, $P<0.01$) than the parameters estimated independently for each treatment (as indicated in the Table).

receptor number. Using a two-site model, most binding sites were in the high affinity form (92%) in the cells untreated with DSLET. The fraction of sites in the high affinity form was reduced significantly at 1 hr (47%) and even more markedly at 6 hr (11%) of DSLET pretreatment. Compared to the very rapid reduction in the fraction of binding sites in high affinity form, the down regulation of total DSLET binding sites ocurred relatively slowly. DSLET (1 μM) pretreatment for 1, 6 and 24 hr reduced receptor density by about 0% (no significant change), 78%, and 90%, respectively.

3. Chronic opioid effect on the density of dihydropyridine-sensitive Ca^{2+} channels

There are reports that chronic opioid treatment may change the binding of [^{3}H]nifedipine in neuronal cell membranes (Ohnishi, 1988, 1989; Inoki, 1990). Since Ca^{2+} enters ND8-47 cells through dihydropyridine-sensitive Ca^{2+} channels after DSLET treatment (Tang et al., 1994), it was possible that a decrease in Ca^{2+} channel density or affinity might contribute to the desensitization of the opioid response resulted from chronic opioid treatment. [^{3}H]nitrendipine binding assays were conducted in membranes of untreated ND8-47 cells and of cells pretreated with 1 μM DSLET for 24 hr. Table 6 shows that there is no significant change of [^{3}H]nitrendipine binding site density or affinity in ND8-47 membranes after DSLET treatment. The K_i values for nitrendipine binding in untreated cells or DSLET-treated cells were 0.89 and 0.66 nM respectively; both values are close to the K_i value (0.49 nM) for nitrendipine binding in hippocampal tissue reported by Ohnishi et al.

TABLE 6

Hours of DSLET (1μM) pretreatment	No. of independent assays	Ki (%CV) (nM)	B_{max} (%CV) (fmol/mg protein)
Control	3	0.89 (30%)	117 (25%)
24	3	0.66 (43%)	130 (35%)

Competition for [^{3}H]nitrendipine (1 nM) binding by unlabeled nitrendipine in membranes from control and treated ND8-47 cells exposed to DSLET for 24 hr.
B_{max} and K_i values were estimated from the pooled data by non-linear regression (ligand) in a one-site model. The values are means (%CV) of three independent experiments. %CV is a percentage coefficient of variation (standard error divided by the parameter value, see Munson & Rodbard, 1980). DSLET treatment did not significantly alter the parameter values.

(1991).

4. G proteins in ND8-47 cells; effects of chronic opioid treatment

Possible changes in the concentration of G proteins during chronic activation of the opioid receptor in ND8-47 cells by DSLET were examined by Western blot analysis. The presence of G protein α subunits ($G\alpha_i$, $G\alpha_o$, $G\alpha_q$, and $G\alpha_s$) were examined in membranes from untreated ND8-47 cells (Fig. 27 A), or from ND8-47 cells chronically treated with DSLET for 24 hr (Fig. 27 B). G protein α subunits α_{i2} (40 kDa), α_{i3} (41 kDa), αs (45 kDa), and α_q (41 kDa) were detected; α_{i1} (41 kDa) and α_o (39 kDa) were not detected in ND8-47 cell membranes. DSLET-treatment of ND8-47 cells for 24 hr did not alter the apparent concentration of the α-subunits assayed in cell membranes.

5. Summary

The results from chronic DSLET treatments in ND8-47 cells reveal a process of desensitization of opioid receptor-mediated increase in $[Ca^{2+}]i$, which is associated with a relatively rapid reduction of receptor affinity for agonist with a decrease in the ratio of high affinity sites/low affinity sites, and a slower reduction of receptor density. The reduction of receptor affinity may be resulted from the uncoupling of receptor with G protein, as GTPγS and PTX also reduce the receptor affinity.

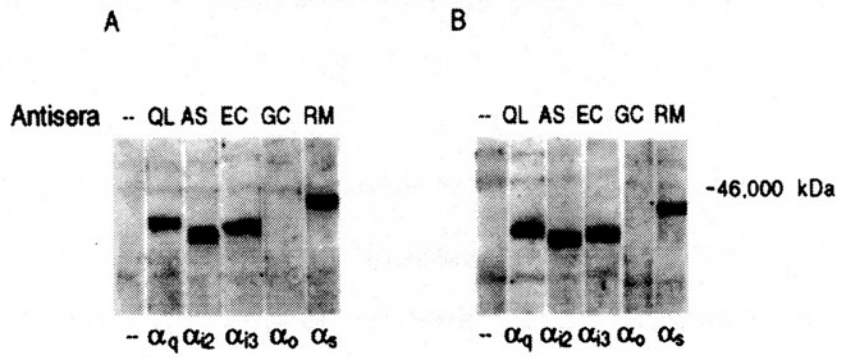


Fig. 27. Western blot of G protein α-subunits in membranes of untreated ND8-47 cells (A), or ND8-47 cells treated with 1 μM DSLET for 24 hr (B).
G proteins from cell membranes (20 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose and incubated with the following antisera: QL (selective for α_q) at 1:500; AS (selective for α_{i1} and α_{i2}) at 1:4,000; GC (selective for α_o) at 1:1,000; EC (selective for α_{i3}) at 1:1,000; and RM (selective for α_s) at 1:4,000. This is a representative record from an experiment conducted three times with similar results.

Section IV
Opioid Regulation of $[Ca^{2+}]i$ in Cultured Mouse Dorsal Root Ganglion Neurons

Although the established neuronal cell lines (*e.g.* NG108-15, 7315c, ND8-47) have been successfully used as cellular models to study opiate effect, one of the major concerns is whether these cells truly reflect physiological neuronal functions. In most circumstances, the results observed in a cell line need to be confirmed with studies in *vivo* (animal), or in *vitro* using neuronal tissues or primary cultures of neuronal cells. Cultured mouse dorsal root ganglion (DRG) neurons are more closely parallel the functions of normal neurons in *vivo*..

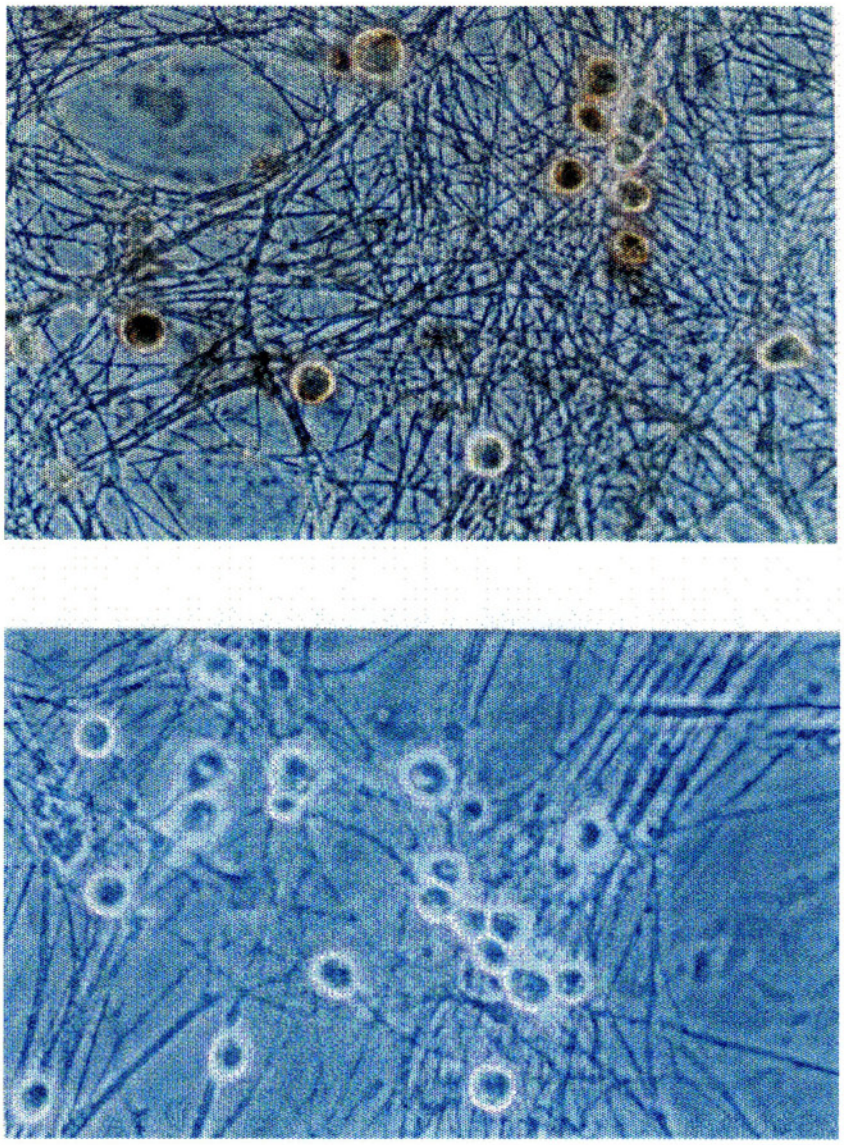
DRG neurons are involved in somatic sensory transduction, such as that for pain, temperature, and proprioception. All three types of opioid receptors (μ, δ, and κ) have been found in DRG neurons (Werz and MacDonald, 1985; Jia and Nelson, 1987; Schroeder and McCleskey, 1993). The sensory neurons within DRG have been divided into large light neurons and small dark neurons on the basis of their cytoplasmic appearance and perikaryal size (Scott, 1992). This is of interest because neurons with different perikaryal size may have distinct axon diameters and subserve diverse functions. There are also histochemical differences in dorsal root ganglion cells. Different populations of DRG neurons show distinct sensitivity to receptor agonists, such as capsicin, adenosine, bradykinin, ATP, and substance P (Bowie et al., 1994). So far, our knowledge is still very limited with regard to opioid receptor distribution and the functions

they regulate in subpopulations of DRG neurons. In this study, we examined opioid effects on $[Ca^{2+}]i$ in subpopulations of mouse embryonic DRG neurons in culture. We found that opioids increase resting $[Ca^{2+}]i$ predominantly in larger diameter DRG neurons, while in smaller diameter DRG neurons the predominant action is inhibition of K^+-stimulated increase in $[Ca^{2+}]i$.

1. Distribution of cell size and expression of substance P-like immunoreactivity

After culturing DRG cells for 10 days, the non-neuronal background cells (glia) were almost totally eliminated. DRG neurons were easily identified by round phase bright cell bodies with a few (usually only 1 or 2) processes, and sharply defined nuclei and nucleoli. The presence of the neurotransmitter, substance P, in these neurons can be demonstrated immunohistochemically. In Fig. 28 A, the neuronal cell bodies with brown color represent the expression of substance P-like immunoreactivity (SP-LI). In a control treatment with substance P-absorbed antiserum, SP-LI was not detected (Fig. 28 B). The distribution and frequency of the DRG neurons with differing somatic diameters were determined in 264 randomly selected neurons cultured for 2 weeks (Fig. 29). The range of cell size is observed from 10 μm to 40 μm. The majority of neurons in each culture had a somatic diameter of 16-25 μm. The expression of SP-LI was detected in 48% of the measured neurons. These were predominantly the small- and intermediate-sized neurons (diameter < 26 μM) although a small fraction of larger-sized neurons also expressed SP-LI (Fig. 29). This frequency and

A



B

Fig. 28 Detection of substance P in cultured DRG neurons by immunochemical assay.
Cultures of DRG neurons were fixed with paraformaldehyde. The cells were then incubated with rabbit antiserum (dilution 1:500) directed against substance P overnight at room temperature (A); or incubated overnight with the same antiserum pretreated for 24 h with 10 μM substance P (B). The procedures are described in detail in the methods.

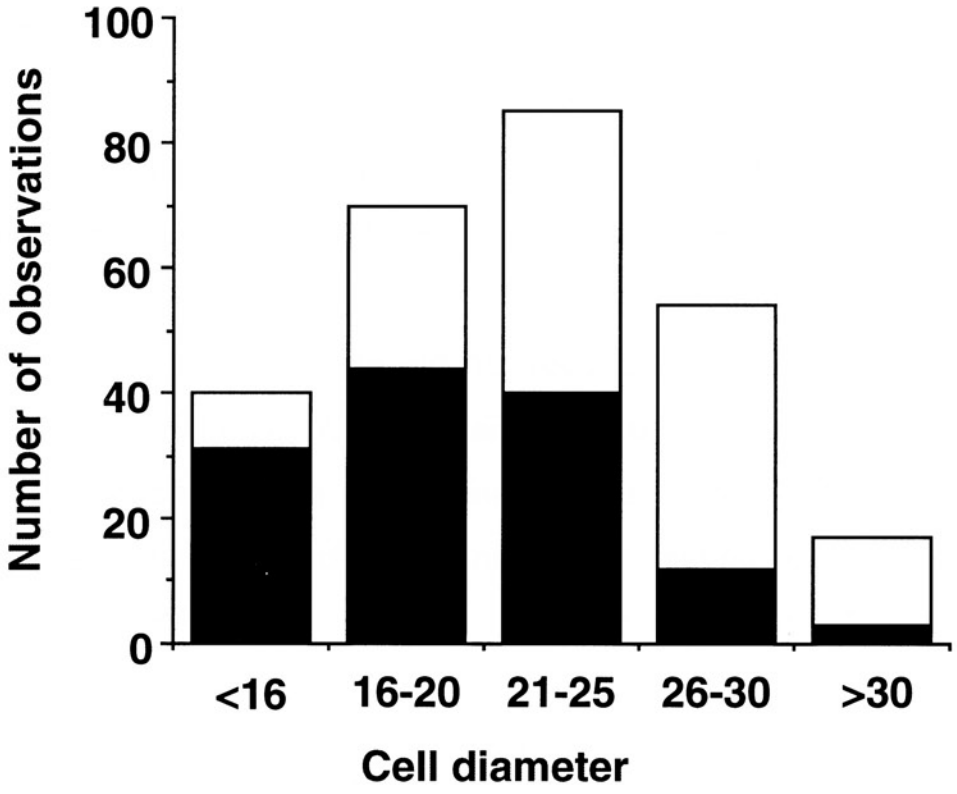


Fig. 29. Distributions of the perikaryal size and of expression of substance P-like immunoreactivity (SP-LI) in DRG neurons. The immunocytochemistry was performed in three similar cultures (three culture dishes), and the presence or absence of SP-LI was determined in all neurons in culture. The somatic diameter of neurons was measured using a graticule eye-piece (no obvious cell shrinkage was observed after fixation). The expression of SP-LI (shaded bar) was detected in 48% (128/264) of randomly selected total 264 neurons.

distribution are close to that in other DRG neuron preparations such as in neonatal rat and cat DRG neurons (Scott, 1992; Bowie et al. 1994).

2. Opioid induced increase in $[Ca^{2+}]i$

To test opioid effects on $[Ca^{2+}]i$, DSLET (which preferentially acts at δ receptors), DAMGO (selective μ agonist), and U69593 (selective κ_1 agonist), were used in concentrations from 100 nM to 10 μM. These agonists did not reduce the resting level of $[Ca^{2+}]i$ in the DRG neurons. A small fraction (<30%) of neurons responded to opioid agonists with an transient increase in $[Ca^{2+}]i$, in a concentration-dependent manner. The resting level of $[Ca^{2+}]i$ was 117 ± 2 nM (A total 538 neurons were studied in 16 separate experiments). For the cells showing an increase in $[Ca^{2+}]i$ induced by opioids, the average resting level of $[Ca^{2+}]i$ was 121 ± 5 nM (n=71). The intracellular calcium level usually increased immediately after adding opioid, reached a peak level within 20 to 30 sec and then returned to baseline within one and a half min. The percentages of neurons responding to DSLET, U69593, and DAMGO at three different concentrations (100 nM, 1 μM, and 10 μM) are indicated in Fig. 30. Even at 10 μM, the DSLET increased $[Ca^{2+}]i$ in only about 27% of recorded cells. The amplitudes of increases in $[Ca^{2+}]i$ induced by each concentration of opioids were not completely analyzed. The maximum amplitude of increase in $[Ca^{2+}]i$ induced by 10 μM DSLET was about 74% above the basal level. The rank order for the occurrence of responding cells is: DSLET (δ) > U69593 (κ) > DAMGO (μ). In cells pretreated with 1 μM naloxone, we did not

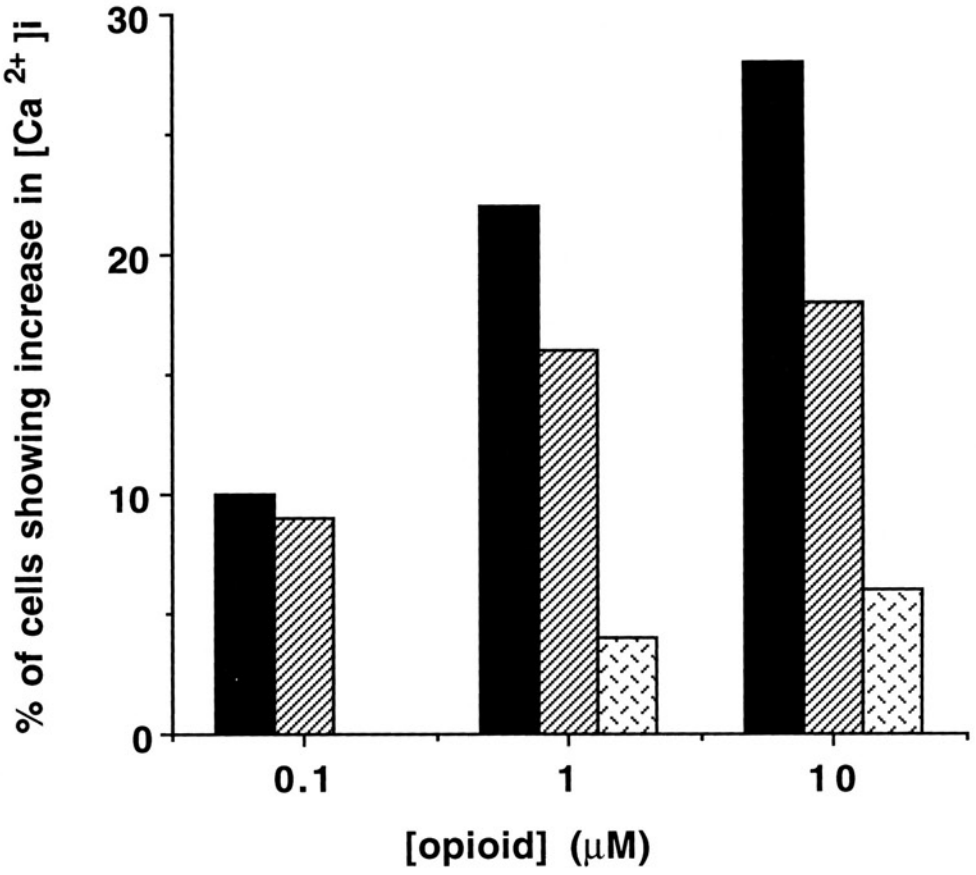


Fig. 30. Incidence of DRG neurons showing an increase in $[Ca^{2+}]i$ induced by opioid agonists.
Percentage of cells responding (with an increase of more than 100 fluorescence intensity units) to DSLET (■), U69593 (▨), or DAMGO (⊠) at concentrations of 0.1 μM, 1 μM, and 10 μM. The total number of cells recorded at each concentration of opioids were between 40 and 80 (3-5 separate cultures).

observe an increase in $[Ca^{2+}]i$ induced by 1 μM DSLET, U69593, or DAMGO. To explore the source of opioid induced increase in $[Ca^{2+}]i$, we treated DRG neurons with dihydropyridine-sensitive calcium channel blocker, nifedipine (1 μM), about 1 min before they were challenged with 1 μM DSLET. In these conditions, we did not observe a DSLET-induced increase in $[Ca^{2+}]i$ in any cell (n=60 cells).

We also examined the frequency of DSLET (1 μM)- or U69593 (1 μM)-induced increase in $[Ca^{2+}]i$ in subsets of DRG neurons with differing somatic size (Table 7). We arbitrarily divided DRG neurons into three groups according to their somatic diameter: small-sized (<16 μm), intermediate-sized (16-25 μm), and large-sized (>25 μm). Opioid-induced increase in $[Ca^{2+}]i$ was mainly observed in large-sized neurons with 7 out of 15 for DSLET (1 μM) and 3 out of 14 for U69593 (1 μM) respectively. In intermediate-sized neurons, 2 out of 16 responded to 1 μM DSLET or U69593 with an increase in $[Ca^{2+}]i$. In small-sized neurons, only 1 out of 14 responded to 1 μM DSLET, and none of the 16 neurons responded to 1 μM U69593.

3. Opioid-induced inhibition of K^+-stimulated increase in $[Ca^{2+}]i$

Although not observing a decrease in resting level of $[Ca^{2+}]i$ induced by opioid agonists, we found that opioid agonists inhibit the increase in $[Ca^{2+}]i$ induced by an elevated concentration of KCl (25 mM). We measured the average peak level of K^+-stimulated increase in $[Ca^{2+}]i$ in individual cells pretreated with 1 μM DSLET, U69593, or DAMGO. As indicated in Fig. 31, K^+ (25 mM) increased the $[Ca^{2+}]i$ from the basal level of 117 nM to 880 nM (about 650% increase). This increase in $[Ca^{2+}]i$ was significantly reduced when the cells were

TABLE 7

Neuronal size	DSLET (1μM)	U69593 (1μM)
Small (<16μm)	1/14	0/16
Intermediate (16-25μm)	2/16	2/16
Large (>25μm)	7/15	3/14

Incidence of DSLET- or U69593-induced increase in $[Ca^{2+}]i$ in DRG neurons with differing size.

Before measurement of $[Ca^{2+}]i$, the neurons were deliberately selected from 3 to 4 separate cultures according to their somatic diameter measured using a graticule eye-piece. 14 to 16 cells were used for each treatment in each group of neurons (small, intermediate, and large).

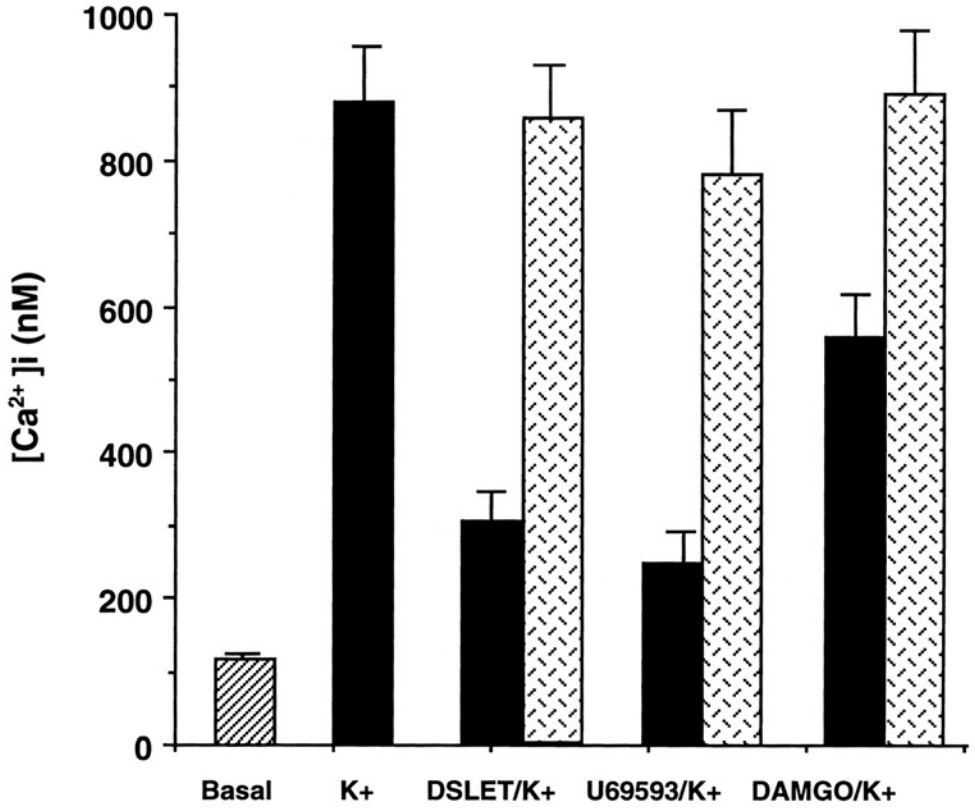


Fig. 31. Suppression of 25 mM KCl-stimulated increase in $[Ca^{2+}]i$ by opioid agonists.

The basal level of $[Ca^{2+}]i$ (▨) or peak level of K^+-stimulated increase in $[Ca^{2+}]$ (■) was measured in untreated cells (K^+) or cells treated with 1 μM DSLET (DSLET/K^+), U69593 (U69593/K^+), or DAMGO (DAMGO/K^+) for 60 to 90 sec. Naloxone antagonism (▨) was measured by treating cells with 1 μM naloxone for 1 min before adding opioid agonist. The number of cells recorded for each treatment were between 40 to 80 (3-5 separate cultures).

pretreated with opioid agonist, DSLET, U69593, or DAMGO; the peak level of $[Ca^{2+}]i$ were 316 nM, 251 nM, and 563 nM respectively in the presence of both K^+ and the opioid. The inhibitory effects of DSLET, U69593, and DAMGO were blocked by pretreatment of cells with 1 μM naloxone (Fig. 31).

DRG neurons with differing somatic diameter presented different sensitivity to opioid inhibitory effects on the K^+-stimulated increase in $[Ca^{2+}]i$. In the three groups of cells, 25 mM K^+ induced a similar increase in $[Ca^{2+}]i$ (900 nM). This increase was strongly inhibited by 1 μM DSLET or U69593 in small-sized neurons with the peak level being 182 nM and 164 nM respectively in the presence of opioid. In contrast, in large-sized neurons K^+ (25 mM) increased $[Ca^{2+}]i$ by 487 nM and 450 nM in the presence of DSLET, or U69593, respectively (Fig. 32).

4. Summary

This study shows that opioids regulate intracellular calcium with different manners in subpopulations of DRG neurons with differing somatic diameter. The small- and intermediate-sized neurons are more sensitive than large-sized neurons to the opioid inhibition of K^+-stimulated increase in $[Ca^{2+}]i$. Substance P is also more frequently detected in small- and intermediate-sized neurons. In contrast, the large-sized neurons are more sensitive than small- and intermediate-sized neurons to the opioid-induced increase in $[Ca^{2+}]i$. In neurons showing opioid-induced increase in $[Ca^{2+}]i$, no significant opiate inhibition of K^+-stimulated increase in $[Ca^{2+}]i$ is observed. These results imply that the DRG neurons showing

different response to opioid constitute distinct neuronal populations and may play different roles in physiological or pathological reactions.

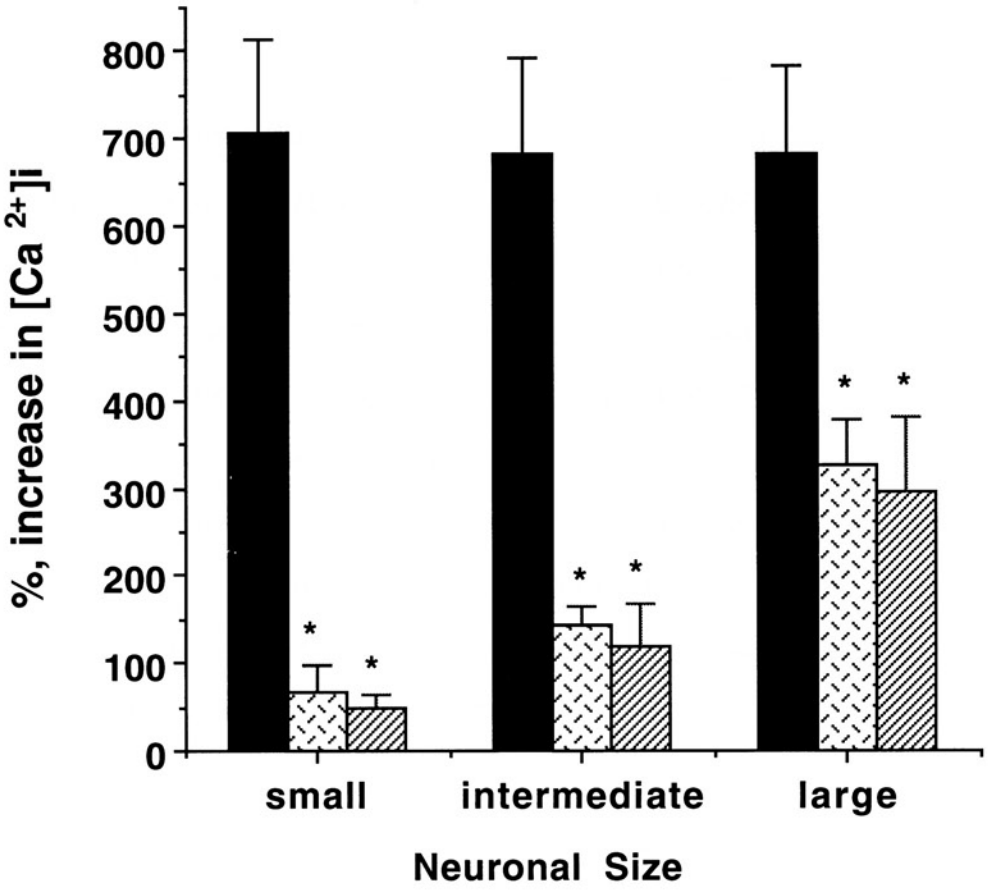


Fig. 32. Suppression of 25 mM KCl-stimulated increase in $[Ca^{2+}]i$ by 1 μM DSLET or U69593 in neurons with differing size. 14 to 16 untreated cells (■) and cells treated with DSLET (⊡) or U69593 (▨) were used for each group of neurons (small, intermediate, and large) deliberately selected from 3 to 4 separate cultures according to their somatic diameter (* $P < 0.05$).

DISCUSSION

I. Opioid Receptor and Its Regulation of $[Ca^{2+}]i$ in ND8-47 Cells

Like the commonly used neuronal cell line NG108-15, ND8-47 cells express only the δ-type opioid receptor. The receptor density (200,000 sites/cell) is close to that in NG108-15 cells (300,000 sites/cell) (Klee, 1974). The characteristics of ND8-47 cells have not been thoroughly studied yet (e.g. other receptors expressed by this cell line and its sensory neuron properties have not been characterized). Other closely related cell lines, ND8 and ND8-34, have been shown to respond to two chemical activators of nociceptive sensory neurons, bradykinin and capsaicin (Wood, et al. 1990). The binding assays indicate that ND8 and ND8-34 cell lines also contain relatively high amount of δ-receptors.

The δ-receptor agonists induced a transient increase in $[Ca^{2+}]i$ in ND8-47 cells. This concentration-dependent effect is due both to an increased fraction of cells responding to opioids and also to an increased $[Ca^{2+}]i$ per cell at higher opioid concentration. In respect of the δ receptor subtypes involved in the opioid induced response in ND8-47 cells, although δ-receptor agonists DSLET, DPDPE and deltorphin-II showed similar receptor binding affinities, their potencies in increasing $[Ca^{2+}]i$ were different. Both DSLET and deltorphin-II were much more potent than DPDPE. This difference results mainly from the activation by DSLET or deltorphin-II of a higher fraction of cells than the fraction activated by DPDPE. Because DPDPE and deltorphin-II have δ_1 and δ_2 selectivity respectively, and DSLET is at least partially selective for δ_2 site, it

is suggested that δ_2-type opioid receptors are mainly involved in increasing $[Ca^{2+}]i$ in ND8-47 cells. This observation was supported by studies (Fig. 6), where the δ_2 specific antagonist, NTB (Sofuoglu et al., 1991, 1993) showed much higher potency and a significantly greater maximum inhibitory effect than the δ_1-specific antagonist, BNTX (Sofuoglu et al., 1991, 1993). We do not know if the smaller fraction of cells responding to DPDPE carry δ_1 receptors capable of mediating an increase in $[Ca^{2+}]i$, or if they have a larger receptor reserve of the δ_2 type receptor, allowing DPDPE to function as an δ_2 receptor agonist in these cells, but with lower efficacy than that of DSLET.

Studies on opioid excitatory effects in NG108-15 cells or DRG neurons have revealed several mechanisms for opioid-induced increases in $[Ca^{2+}]i$ or Ca^{2+} conductance. In NG108-15 cells, the δ-agonist evoked increase in $[Ca^{2+}]_i$ resulting either from a Ca^{2+} influx in differentiated cells or a Ca^{2+} release from the inositol 1,4,5-triphosphate (IP_3)-sensitive store in undifferentiated cells; these effects were blocked by pertussis toxin but not by cholera toxin, indicating a G_i- or G_o-mediated effect (Jin et al., 1992; 1994). In DRG cells, cholera toxin-A or -B subunits, as well as the whole toxin, selectively blocked the opioid-induced prolongation of the Ca^{2+} component of action potential (Shen and Crain, 1990). Opioid facilitatory, but not inhibitory, modulation of the action potential was prevented by injection of an inhibitor of cyclic adenosine monophosphate (AMP)-dependent protein kinase into DRG neurons (Chen et al., 1988). Furthermore, opioids can stimulate basal adenylyl cyclase activity in these cultures (Crain et al., 1988).

Based upon these observations, it was proposed that opioid-induced action potential prolongation in DRG neurons is mediated by opioid receptor subtypes that are positively coupled via G_s to adenylyl cyclase/cyclic AMP-dependent voltage-sensitive ionic conductances (Shen and Crain, 1989; 1990).

The biochemical basis for opioid-induced increase in $[Ca^{2+}]i$ in ND8-47 cells appeared different from that previously proposed in the studies with NG108-15 cells or DRG neurons. Using Western analysis of G protein α subunits in ND8-47 cell membranes, we found that α_{i2}, α_{i3}, α_s, and α_q were present; however, α_o and α_{i1} were not detectable. This result is aparently different from that in other neuronal cell lines that have opioid receptors such as NG108-15 cells (δ-receptor) and 7315c pituitary tumor cells (μ-receptor). In other experiments, the GC antiserum clearly identified a ~39 kDa band (believed to be α_o) in both NG108-15 membranes (Tang et al., submitted) and in 7315c membranes (Izenwasser and Cote, 1995). In addition, antiserum AS (selective for α_{i1} and α_{i2}) clearly identified a doublet (apparent molecular weights of 41 and 40 kDa) in 7315c membranes (Goldsmith et al., 1987) but in NG108-15 and ND8-47 membranes only the lower 40 kDa band (believed to be α_{i2}) was detected. It is possible that the lack of α_o (which is believed to mediate inhibition of Ca^{2+} channels) in ND8-47 cell membranes is the reason that DSLET only opens Ca^{2+} channels in these cells, while in NG108-15 cells, which have both α_{i2} and α_o, δ agonists at lower concentrations open Ca^{2+} channels (probably through α_{i2}) but at higher concentrations predominantly activate α_o and close Ca^{2+} channels. Studies using an antisense oligonucleotide treatment

approach supported the assumption that $G\alpha_{i2}$ is required for opioid-induced increase in $[Ca^{2+}]i$ in ND8-47 cells.

Although there are structural differences of the α subunits among various G proteins, they have high level of conservation. The sequences of α_{i2} and α_{i3} antisense oligonucleotides used in our study (as used by Gollasch et al.,1993), are complementary to translated regions of α_{i2} and α_{i3} mRNA showing very limited homology. Considering that unmodified phosphodiester oligodeoxyribonucleotides are unstable in biological fluids and display poor cellular uptake characteristics (Steinand and Cohen, 1988; Helene and Toulme, 1990), the antisense oligonucleotides were modified with phosphorothioate groups at the two ends of each of the oligonucleotide. The $G\alpha_{i2}$ antisense oligonucleotide (α_{i2}-AS) markedly inhibited the expression of $G\alpha_{i2}$ and significantly blocked the DSLET-induced increase in $[Ca^{2+}]_i$ in ND8-47 cells. α_{i2}-AS had no effect on the expression of other α subunits. Treatment of the cells with antisense oligonucleotides to $G\alpha_{i3}$ and $G\alpha s$, or with sense oligonucleotide for $G\alpha_{i2}$, had no significant effect on the opioid response. The inhibition of opioid response by the $G\alpha_{i2}$ antisense treatment appears to be related to a reduction in the amount of $G\alpha_{i2}$ protein in ND8-47 cell membranes since this treatment reduced the level of $G\alpha_{i2}$ but had no effect on the levels of $G\alpha_q$, $G\alpha_{i3}$, and the two $G\alpha_s$ subunits. Treatment of ND8-47 cells with antisense oligonucleotides against $G\alpha_{i3}$ or $G\alpha_s$ reduced the levels of these G proteins but did not influence the DSLET-induced increase in $[Ca^{2+}]i$.

By treating ND8-47 cells with phosphorothioate antisense

oligonucleotides, our studies clearly demonstrate that α_{i2} is responsible for opioid-induced increase in $[Ca^{2+}]_i$ in ND8-47 cells. We suggest that it is the G protein coupling to opioid receptors that determines the direction of modulation (opening or closing) of calcium channels by opioids; α_o mediates the inhibition of Ca^{2+} channels while α_{i2} mediates the opening of Ca^{2+} channels. Our studies extend the range of G protein-mediated transduction systems demonstrated to be influenced by opioid receptor activation. In different systems, it is now established that $G\alpha_{i2}$ mediates opioid-inhibition of adenylyl cyclase activity (McKenzie and Milligan, 1990) and activation of dihydropyridine-sensitive calcium channels (Tang et al., 1994), and that $G\alpha_o$ mediates opioid-inhibition of N-type calcium channels (Kleuss et al., 1991). Pertussis toxin-sensitive G proteins also mediate opioid-induced mobilization of Ca^{2+} from intracellular stores (Jin et al., 1994) and the opening of K^+ channels induced by opioids (North and Williams, 1987), but the specific G protein α-subunits implicated in these actions are not yet established.

How do G proteins interact with calcium channels? Based on the current knowledge, there are several possibilities: (1) direct interaction between G-protein and calcium channels by a membrane-confined mechanism; (2) coupling of G protein with receptor activates cytosolic messengers, such as cAMP, IP_3, DG, or induces the release of intracellular calcium which further activates phospholipases to modify the calcium channels; (3) opening of Ca^{2+} channels is induced by the depolarization of cell membrane due to either opening of Na^+ channels or closing of K^+ channels. It is

unlikely that the activation of adenylyl cyclase and the elevation of cAMP are involved in opioid-induced increase in $[Ca^{2+}]i$ as recent preliminary data (Franazek, unpublished observations, 1994) indicate that δ-agonists inhibit adenylyl cyclase in ND8-47 cells. There is no support for opioid-induced mobilization of internal Ca^{2+} through Ca^{2+} store, since TMB-8, known as a blocker of the Ca^{2+} channels on the endoplasmic reticulum, did not affect opioid-induced increase in $[Ca^{2+}]i$ in ND8-47 cells. The protein kinases, PKA and PKC, are also not likely involved in this response as neither the PKA inhibitor, KT 5720, nor the PKC inhibitor, calphostin C, influenced the opioid-induced response. In contrast to the observation in NG108-15 cells, which show that opioids induce membrane depolarization by opening of Na^+ channels and further activate Ca^{2+} influx through voltage dependent Ca^{2+} channels (Jin et al., 1992), our observation in ND8-47 cells suggests that activation of Na^+ channels is not involved in opioid-induced increase in $[Ca^{2+}]i$ because treatment with the Na^+ channel blocker, tetrodotoxin or removal of extracellular Na^+ did not block the opioid response (Tang et al., 1994). Thus, in the process of opioid induced increase in $[Ca^{2+}]i$, it is possible that the activation of δ-receptors and G proteins directly affects the functional state of the L-type Ca^{2+} channels, an effect similar to the L-type Ca^{2+} channel activation by the specific agonist Bay K 8644 (Dolphin and Scott 1988, 1989).

The studies of chronic opioid effects on opioid regulation of intracellular Ca^{2+} indicate the occurrence of two time-dependent processes occur during sustained exposure of ND8-47 cells to DSLET: (1) desensitization, characterized as the loss in the ability of opioid

agonists to inhibit adenylyl cyclase activity, and (2) down-regulation, defined as the decrease in the number of binding sites. There was no significant change in receptor density after 1 hr of DSLET treatment at which time the agonist response was significantly attenuated. However, after 6 hr of 1 μM DSLET exposure, there was a significant decrease in the receptor density. Chronic DSLET treatment in ND8-47 cells induces a relatively faster desensitization and receptor down-regulation compared to the chronic morphine exposure in 7315c cells, in which a significant down-regulation did not occur until 24 hr of 100 μM morphine exposure (Puttfarcken et al., 1988). Again, our binding data show that, after desensitization has occurred, there is no significant change in estimates of K_i(H) and K_i(L), but a significant ($P<0.05$) increase in fraction of binding sites in the low affinity form, and at longer time intervals a reduced total number of binding sites. In aggreement with these findings, our binding data demonstrated that the exposure of cells or cell membranes to pertussis toxin or GTPγS shifted the binding sites for DSLET to a low-affinity state without any changing in total receptor number. The similarities in both the functional consequence and binding results between pertussis toxin treatment and chronic opiate treatment confirm the view that one of the primary events in opioid tolerance is uncoupling of receptors from G proteins (Costa et al., 1983; Puttfarcken et al., 1988; Werling et al., 1989)

The results from chronic opioid treatment of ND8-47 cells contrast sharply with observations in rat sensory neurons (Crain and Shen, 1992), in which chronic opioid exposure induced

supersensitivity to the G_s-coupled excitatory effects of opioids, suggesting that the opioid-induced prolongation of Ca^{2+}-channel dependent action potential duration (APD) is not desensitized by the chronic opioid treatment. The absence of desensitization of excitatory opioid receptor function during sustained agonist binding was explained as possibly due to a deficiency in certain receptor kinase or arrestin-like molecules which mediate the homologous desensitization that occurs in β-adrenergic receptors (Crain and Shen, 1992).

In addition to a change in G protein coupling state under chronic opioid treatment, opioid-induced changes in the concentrations of G protein subunits have been reported in certain mammalian tissues. However, alterations in G protein concentration during sustained opioid exposure are not consistently observed. Attali and Vogel (1989) found that chronic treatment of rat dorsal root ganglion-spinal cord preparations with the κ-agonist U50488H for 4 days reduced the level of $G_{\alpha i1}$ by about 30%. In the same system, the μ-agonist DAMGO was reported to reduce both $G_{\alpha i}$ and $G_{\alpha o}$ levels, while chronic treatment with the δ-agonist, DPDPE, increased the levels of the same G protein subunits (Vogel et al., 1990). Treatment of NG108-15 cells with DADLE or morphine for up to 72 h did not alter the concentration in either $G_{\alpha o}$ or $G_{\alpha i2}$ measured immunologically (Lang and Costa, 1989). Our result also failed to show any significant change of the concentration of G protein subunit after chronic DSLET treatment. Thus the desensitization and receptor down regulation in ND8-47 cells are not necessarily accompanied by changes in the levels of G protein

subunits.

Another possible mechanism involved in the desensitization of opioid response in ND8-47 cells is an increase in the number of calcium channels or an increase in the Ca^{2+} affinity for the opioid-regulated Ca^{2+} channels. It has been reported that chronic morphine treatment of rats increased the [^{3}H]dihydropyridine binding sites in hippocampal slices and also shifted the binding to a high affinity state (Ohnishi et al., 1988, 1989, 1991). Activation of Ca^{2+} channels by L-type Ca^{2+} channel agonist Bay K-8644 is blocked by pertussis toxin and potentiated by GTPγS in cultured rat DRG cells (Dolphin and Scott, 1988, 1989), suggesting an intimate interaction between G protein and L-type Ca^{2+} channel. In this study, it has been shown that opioids activate dihydropyridine-sensitive Ca^{2+} channels by a PTX-sensitive mechanism. Estimates of [^{3}H]nitrendipine binding after DSLET treatment of ND8-47 cells indicate that DSLET pretreatment did not change the binding affinity or the number of [^{3}H]dihydropyridine binding sites in ND8-47 cells membranes.

II. Opioid Regulation of $[Ca^{2+}]i$ in Cultured Mouse DRG Neurons

When testing the opioid effects on the resting level of $[Ca^{2+}]i$ in individual DRG neurons, we did not observe a decrease in the $[Ca^{2+}]i$; in a small fraction (less than 30%) of neurons, we observed a transient increase in $[Ca^{2+}]i$ induced by opioid agonists. Using the same technique, in ND8-47 cells, it has been shown that δ agonists induced a transient increase in $[Ca^{2+}]i$ in about 80% of the cells (Tang et al., 1994). This effect was thought to be due to Ca^{2+} influx

through dihydropyridine-sensitive Ca^{2+} channels (Tang et al., 1994) and mediated by a G protein, $G\alpha_{i2}$ (Tang et al., submitted). Although the incidence of DRG cells responding to opioid stimulation is much lower than that observed in ND8-47 cells, some similarities can still be noticed for opioid-induced increase in $[Ca^{2+}]i$ in both DRG neurons and ND8-47 cells. First, the elevation in $[Ca^{2+}]i$ by opioids is transient, moderate, and concentration-dependent in both cases. Second, nifedipine blocked the increase in $[Ca^{2+}]i$ induced by opioid, suggesting a pathway of Ca^{2+} influx through dihydropyridine-sensitive calcium channels. It seems probable that the same mechanism(s) is applied to this opioid-induced increase in $[Ca^{2+}]i$ for both DRG neurons and ND8-47 cells. In DRG neurons, the opiate agonists, DSLET, U69593, and DAMGO, all induced an increase in $[Ca^{2+}]i$ in a small fraction of neurons with a rank order: DSLET (preferentially acts at δ) > U69593 (κ) > DAMGO (μ). Since DSLET mainly acts at δ receptor with a minor action at μ receptors, and DAMGO and U69593 only act at μ and κ receptors, respectively, it suggests that δ receptors may be the main opioid receptors responsible for the increase in $[Ca^{2+}]i$. In ND8-47 cells (where δ receptors are the predominantly opioid receptor type), only δ agonists induced an increase in $[Ca^{2+}]$.

The small fraction of neurons responding to opioids with an increase in $[Ca^{2+}]i$ suggested that there might be subpopulations of neurons responding to opioids very differently. Based on the current knowledge, the discrepancy among DRG neurons is not only on their different size, but more importantly remains in their histochemical and physiological characters. For instance, substance P and CGRP

were more frequently found in small- and intermediate-sized neurons than in large-sized neurons (Scott, 1992). A recent report indicated that the increase in $[Ca^{2+}]i$ elicited by substance P and bradykinin was more frequently found in small- and intermediate-sized DRG neurons (Bowie et al., 1994). In contrast, there is less information about the large-sized neurons although cholera toxin B subunit was mainly detected in large-sized neurons (Scott, 1992). In our study, an opioid-induced increase in $[Ca^{2+}]i$ was mainly observed in large-sized neurons, and only occasionally in intermediate- or small-sized neurons. This action of opioids may not be involved in their analgesic effect since the large-sized neurons mainly represent the A fiber neurons with thick myelinated axons which are thought to convey proprioceptive sensations (Scott, 1992). Yet, it has been noticed in clinical practice that several opioids can cause rigidity of trunk muscles; this effect is believed to be the result of an action of the opioids at spinal level (Yaksh and Noueihed, 1985). The opioid-induced increase in $[Ca^{2+}]i$ might be involved in opioid-induced muscular rigidity.

To examine the inhibitory effect of opioids on K^+-stimulated increase in $[Ca^{2+}]i$, we measured the mean peak levels of K^+-stimulated increase in $[Ca^{2+}]i$ in neurons with or without prior opioid treatment. The opioid agonists, U69593, DSLET, and DAMGO all inhibited the K^+-stimulated increase in $[Ca^{2+}]i$ in a significant number of DRG neurons. These results suggest that κ_1, δ, and μ receptors can also regulate the K^+-activated Ca^{2+} channels. The rank order for opioid (1 μM) inhibition of 25 mM K^+-stimulated increase in $[Ca^{2+}]i$ was: U69595 $\geq$ DSLET > DAMGO. Although the rank

order of potencies does not reflect the distribution of different opioid receptor types in mouse DRG neurons, it appears that the μ receptors are less active than either δ or κ receptors in the regulation of intracellular calcium or calcium channels in fetal DRG neurons in culture.

The inhibition of K^+-stimulated increase in $[Ca^{2+}]i$ by opioids was much greater in small- sized neurons than in large-sized neurons. This phenomenon may be physiologically significant since substance P was more frequently detected in small- or intermediate-sized neurons. It has been proposed that opioid agonists interact with substance P-containing neurons and inhibit substance P release (Mudge et al., 1979). Substance P has been thought to mediate central transmission of nociceptive stimuli, and opiate blockade of substance P release would serve as a basis for opioid antinociceptive action.

As noted above, the direction and intensity of opioid-induced responses were largely determined by the somatic size of the neurons. Yet, there is still an overlap of opioid responses among different groups of neurons. For example, opioid-induced increase in $[Ca^{2+}]i$ was mainly observed in large-sized neurons but also in a small fraction of intermediate- or small-sized neurons. Again, a striking opioid-induced inhibition of K^+-stimulated $[Ca^{2+}]i$ was observed in small- and intermediate-sized neurons but it was also noticed in large-sized neurons. This overlap possibly resulted from our arbitrary division of neurons into three groups according to their somatic size, but may also suggest that the opioid-induced response is not only determined by the size of neurons.

III. Concluding Remarks

It has been the aim of this project to understand how opioid receptors regulate the intracellular free calcium in sensory neurons. Most of the experiments have been performed in a sensory neuronal cell line, ND8-47, which expresses a high density of opioid δ receptors. Opioids demonstrate an unsusal effect in these cells by inducing an transient increase in the $[Ca^{2+}]i$, resulting from the Ca^{2+} influx through the dihydropyridine-sensitive L-type Ca^{2+} channels. Using an antisense oligonucleotide treatment approach, it has been shown that $G\alpha_{i2}$ protein is required for opioid induced increase in $[Ca^{2+}]i$. Another important feature for these cells is that the $G\alpha_{i2}$ protein is present while $G\alpha_o$ is not detectible in the cell membranes using Western blot. This is in contrast to NG108-15 cells, in which both $G\alpha_{i2}$ and $G\alpha_o$ are present; both excitatory and inhibitory effects of opioids have been observed in these cells. It is suggested that the absence of the $G\alpha_o$ protein (which is believed to mediate inhibition of Ca^{2+} channels) in the ND8-47 cell membrane might be responsible for the lack of inhibition of $[Ca^{2+}]i$ by opioid. The important role of $G\alpha_o$ in the neuronal system was recently proposed in an animal model. In *Caenorhabditis elegans*, it is has been found that loss of $G\alpha_o$ produces hyperactive worms, while worms with too much $G\alpha_o$ activity are sluggish (Mendel et al., 1995). In this sense, the absence of $G\alpha_o$ in ND8-47 cells provides a novel cellular model not only for studying opioid stimulatory effects, but also a useful tool in studing the function of $G\alpha_o$ protein. Insertion of

$G\alpha_o$ into these cells may be a valuable approach in the future studies.

The studies in cultured DRG neurons reveal different patterns of opiate regulation in $[Ca^{2+}]i$, depending upon the perikaryal size. In smaller sized neurons, substance P is most frequently detected; there is a lower incidence of neurons responding to opioids with an increase in $[Ca^{2+}]i$, but greater inhibition of K^+-induced increase in $[Ca^{2+}]i$ by opioids. On the other hand, larger sized neurons, in which substance P is rarely detected, more frequently responded with an increase in $[Ca^{2+}]i$ to application of opioids; in these cells a less degree of inhibition of K^+-induced increase in $[Ca^{2+}]i$ by opioids was observed. Interestingly, the opioid-induced increase in $[Ca^{2+}]i$ has also been demonstrated to be due to the opening of the dihydropyridine-sensitive L-type Ca^{2+} channels, indicating a common ground for this opioid-induced increase in $[Ca^{2+}]i$ in both ND8-47 cells and the DRG neurons. We have not examined the type of Ca^{2+} channels that may be involved in opioid inhibition of K^+-induced increase in $[Ca^{2+}]i$; nor do we know as yet the type of G proteins responsible for opioid induced changes in $[Ca^{2+}]i$ (either increase or decrease) in the DRG neurons.

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